CARACTERIZACIÓN Y DINÁMICA DE LOS PATRONES DE INFECCIONES ÚNICAS Y MÚLTIPLES PARA SEIS TIPOS DEL VIRUS DE PAPILOMA HUMANO DE ALTO RIESGO

SARA CECILIA SOTO DE LEÓN, B.Sc.

DOCTORADO EN CIENCIAS BIOMEDICAS

UNIVERSIDAD NUESTRA SEÑORA DEL ROSARIO

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Presentado por: **SARA CECILIA SOTO DE LEÓN, B.Sc.**

Director: **MANUEL ALFONSO PATARROYO, M.D., Dr.Sc.**

Co- Director: **RICARDO SANCHEZ, M.D., MSc.**

"TESIS DE DOCTORADO PRESENTADA COMO REQUISITO PARCIAL PARA OPTAR AL TITULO DE DOCTOR EN CIENCIAS BIOMÉDICAS"

DOCTORADO EN CIENCIAS BIOMEDICAS

UNIVERSIDAD NUESTRA SEÑORA DEL ROSARIO

BOGOTÁ, D.C. 2014

HOJA DE APROBACIÓN

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MANUEL ALFONSO PATARROYO, M.D., Dr.Sc.

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RICARDO SANCHEZ, M.D., MSc.

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ALBA LUCÍA CÓMBITA, Ph.D.

_____

MARIA FERNANDA GUTIÉRREZ, Ph.D.

_____

SANDRA ROCÍO RAMÍREZ CLAVIJO, Ph.D.

BOGOTÁ, D.C. 2014

DEDICATORIA

A mis abuelos; mis orígenes, mis ángeles…

Y a mis hijos; mi futuro, la extensión de nuestra vida…

AGRADECIMIENTOS

Debo agradecer a Dios porque ha hecho que todo en el universo se encamine a cumplir mis sueños y aumenta mi fe en Él.

A mis padres por su constante motivación, su apoyo incondicional y todo su amor.

A mi esposo mi luz, mi polo a tierra y el amor de mi vida, gracias por tu comprensión y dedicación para que cumpla mis sueños.

A mis hijos que son el motor de todo cuanto hago, mi felicidad y quienes apresuran a acortar mi día.

A mi familia Anto, Lore y Joaquín y mi familia paterna, materna y política, gracias por sus oraciones, por estar tan pendientes de nosotros.

A la familia que escogí: Mile, Mary, Luisa y Andresín, ustedes son los mejores amigos, colegas, confidentes, críticos, revisores y socios que existen!!! Gracias por todo su apoyo académico, profesional y personal.

A Manuel por su paciencia, dedicación y confianza en mí y a Ricardo por su disposición, por escucharme y enseñarme. Ustedes han hecho la profesional que soy ahora. MIL GRACIAS porque he aprendido de los Mejores.

A FIDIC, especialmente al Dr. Manuel Elkin Patarroyo porque ha facilitado que mis sueños se hagan realidad, lo admiro y he aprendido demasiado en esta casa de formación académica y formación de vida.

A mis jurados Doctoras Sandra, Maria Fernanda y Alba Lucía, que han tenido tan excelente disposición para mejorar mi trabajo y a quienes las he podido conocer en otros escenarios donde también he aprendido tanto de ustedes, como profesoras o evaluadoras, gracias por esta preciada y honorosa compañía.

A la Dra. Luisa Matheus quien ha sido parte fundamental de este proceso, su colaboración y apoyo son invaluables.

Al comité doctoral del programa.

A todos las personas que han pasado por nuestro laboratorio en calidad de pasantes por su dedicación y arduo trabajo en cada proyecto que participaron.

CONTENIDO

ABREVIATURAS

CCU. Cáncer de cuello Uterino

VPH. Virus de Papiloma Humano

VPH-AR. Virus de Papiloma Humano de alto riesgo

VPH-BR. Virus de papiloma Humano de bajo riesgo

PCR. Siglas en inglés de Reacción en cadena de la polimerasa, de: *Polymerase Chain Reaction*.

ADN. Acído Desoxirribonucléico.

HMBS. Hidroximetilbilano sintasa.

NIC. Neoplasia Intra Cervical

CHII. Captura Hibrida 2

FDA. Siglas del inglés Food and Drugs Administration

RESUMEN

El cáncer de cuello uterino (CCU) es el tercer tipo de cáncer más común en las mujeres alrededor del mundo, afectando principalmente mujeres en edad fértil (15-44 años). La mayoría de los casos de CCU ocurren en países en vías de desarrollo (principalmente en los continentes de África, Asia y América Latina), como es el caso de Colombia, donde se han reportado tasas de incidencia y mortalidad altas (32,9-36,4 y 18,7 casos/año/100.000 mujeres, respectivamente) y continúa siendo la segunda causa de muerte por cáncer en la población femenina del país.

El principal factor de riesgo para el desarrollo de lesiones cervicales pre-neoplásicas es la infección persistente por ciertos tipos de Virus de Papiloma Humano (VPH) conocidos como de alto riesgo (VPH-AR). Los tipos más comunes de VPH-AR son VPH-16, -18, -45, -31, -33, -52, -58 y -35, y se asocian con ~90% de CCU a nivel mundial. Sin embargo, el 80% de las infecciones causadas por estos tipos son transitorias (desaparecen en 6–8 meses), siendo el 20% restante de infecciones, aquellas responsables de la aparición de las neoplasias intraepiteliales del cérvix (NIC).

Este trabajo tuvo como objetivo identificar las características de la infección por VPH en una población de mujeres socio-demográficamente heterogénea, que habitan en diferentes regiones de Colombia. Para esto, fueron incluidas 2109 mujeres provenientes de las ciudades de Chaparral, Tumaco, Leticia, Bogotá y Girardot, quienes acudieron a los programas de promoción y prevención de CCU implementados en los respectivos hospitales; cada mujer proporcionó información sociodemográfica y de conductas sexuales, además de una muestra de raspado cervical.

Se realizó la determinación de la presencia de VPH por la técnica de reacción en cadena de la polimerasa (PCR), empleando tres juegos de cebadores genéricos (GP5+/6+, MY09/11 y pU1M/2R); adicionalmente, se usaron cebadores tipo-específicos para determinar la frecuencia de seis tipos de VPH de alto riesgo (VPH-AR-16, -18, -31, -33, -45 y -58) y dos de bajo riesgo (VPH-BR-6/11).

Los datos colectados fueron usados para analizar asociaciones entre tipos virales, factores de riesgo, características sociodemográficas y resultados del hallazgo citológico. Del total de las mujeres analizadas, el 49,2% (n= 894) presentaron infección por VPH-AR, de las cuales, el 59,8% (n=583) correspondían a infecciones por más de

un tipo de VPH (infección múltiple), principalmente para las ciudades de Girardot y Leticia. En todas las regiones del estudio, el tipo viral más frecuente fue VPH-16.

Al incluir los datos socio-demográficos en los análisis de asociación, se encontró que el estado marital "Unión Libre" se presentó como un factor de protección, en cuanto a la presencia de infecciones por VPH. En cuanto a otros factores analizados, pertenecer a la ciudad de Girardot y a la etnia indígena son factores de riesgo, los cuales se asociaron adicionalmente con la presencia de coinfecciones por este virus.

Adicionalmente, se evaluó la carga viral mediante PCR en tiempo real y se correlacionaron los datos a través de un seguimiento a dos años, con el fin de determinar la dinámica de los patrones de infecciones únicas y múltiples encontrados en nuestro país, para los seis tipos del VPH-AR de mayor prevalencia en la población colombiana.

Para lo anterior, se cuantificaron los tipos de VPH-AR-16, -18, -31, -33, -45 y -58, y un gen constitutivo celular (HMBS) en muestras de tejido cervical de 219 mujeres, que asistieron mínimo a 4 visitas, con un intervalo de tiempo de 6 meses (± 3 meses).

Las cuantificaciones tipo-específicas de VPH se calcularon tanto a partir del número de copias absolutas presentes en la muestra, como aquellas normalizadas según el gen constitutivo celular, para obtener el número de copias virales por célula. Adicionalmente, se evaluaron las asociaciones con los datos sociodemográficos y clínicos recopilados para este grupo de mujeres, según el tipo viral presente en la muestra.

Se encontró que valores bajos de carga para VPH-31 se asocian con persistencia de este tipo a través del tiempo. De otra parte, bajos valores de carga absoluta para VPH-16, fueron un indicador de pronóstico negativo para el cambio colposcópico (resultado al inicio de la cohorte vs. resultado en el último seguimiento).

Con respecto a los tipos VPH-18 y -31, los resultados mostraron que tienen la menor probabilidad de resolver la infección naturalmente en el tiempo evaluado, y para las infecciones por VPH-16 y -58, las mujeres cuyo origen no es Bogotá, tienen la mayor probabilidad de depurar la infección.

El análisis de la dinámica de las cargas virales para cada uno de los 6 tipos de VPH-AR evaluados, arrojó información sobre los factores que influyen en la disminución de la

carga como lo fueron: el no pertenecer a Bogotá para los tipos VPH-16, -18 y -45, el mayor número de compañeros sexuales para VPH-18 y -45, y la concomitancia con otras infecciones de transmisión sexual diferentes a VIH, para VPH-45.

Los factores que se relacionan con un aumento de la carga viral normalizada son: el uso de cualquier método de planificación familiar para las mujeres infectadas con VPH-16, y el uso de métodos hormonales para VPH-18 y -31; adicionalmente, las mujeres que declararon pertenecer a la etnia blanca aumentaron considerablemente su carga al estar infectadas con VPH-18 o -58. A mayor edad se observó una asociación con aumento en la carga viral para aquellas mujeres infectadas con VPH-31. Para infecciones por VPH-45, la presencia de infecciones múltiples y el antecedente de abortos aumentaron la carga viral en el tiempo.

Se demostró que existen diversos factores de riesgo involucrados en la modulación de la carga viral y que pueden interferir de diferentes formas en cada tipo evaluado; sin embargo, al involucrar la modulación de más de un tipo viral, todos son regulados en el mismo sentido, aumentando o disminuyendo la carga según sea el caso.

Este es el primer estudio en Colombia en realizar la determinación molecular de VPH y la identificación de frecuencias tipo-específicas en varias regiones del país, involucrando características socio-demográficas y poblaciones étnicas diversas, y estableciendo asociaciones de la presencia y carga viral con posibles factores de riesgo. Los resultados destacan la importancia de caracterizar las infecciones por VPH en poblaciones étnicamente diversas, con el fin de ampliar y mejorar el alcance de los programas de promoción y prevención del CCU. Estos resultados brindan información sobre las distribuciones del VPH pre-vacunación, lo que contribuirá con el seguimiento de los planes de vacunación aplicados a las poblaciones colombianas; adicionalmente, estos resultados aportan al conocimiento de la epidemiología del VPH en los países en vías de desarrollo.

SUMMARY

Cervical cancer (CC) is the third type of cancer most commonly affecting women worldwide, chiefly affecting fertile-aged females (15-44 years old). Most CC cases occur in developing countries (mainly on the African, Asian and Latin American continents) like Colombia where high incidence (32.9-36.4 cases/year/100,000 women) and mortality rates (18.7 cases/year/100,000 women) have been reported and continues being the second cause of cancer-related death in Colombia's female population.

The main risk factor for developing pre-neoplastic cervical lesions is persistent infection by certain types of human papilloma virus (HPV), known as high risk types (HR-HPV). The most common HR-HPV types are HPV-16, -18, -45, -31, -33, -52, -58 and -35, these being associated with ~90% of CC around the world. However, 80% of the infections caused by these types are transitory (i.e. they disappear within 6–8 months), the remaining 20% of infections being responsible for the appearance of cervical intraepithelial neoplasia (CIN).

The present work was aimed at identifying HPV infection characteristics in a socio-demographically heterogeneous population of females living in different parts of Colombia; 2,109 women from the cities of Chaparral, Tumaco, Leticia, Bogotá and Girardot were thus included in the study as they were attending CC prevention programmes in their respective hospitals. Every female provided sociodemographic information, data regarding their sexual conduct and a cervical smear.

HPV infection was determined by polymerase chain reaction (PCR) using three sets of generic primers (GP5+/6+, MY09/11 and pU1M/2R); type-specific primers were also used for determining the frequency of six high-risk HPV types (HR-HPV-16, -18, -31, -33, -45 and -58) and two low-risk types (LR-HPV-6/-11).

The data so collected was used for analysing associations between viral types, risk factors, sociodemographic characteristics and cytological findings; 49.2% (n=894) of the females analysed had HR-HPV infection, 59.8% (n=583) of whom were infected by more than one type of HPV (multiple infection), mainly in Girardot and Leticia. HPV-16 was the most frequently occurring viral type in all the regions involved in the study.

When including socio-demographic data in the association analysis it was found that cohabiting as marital state was a protective factor regarding HPV infection. Regarding the other factors analysed, living in Girardot and being indigenous were risk factors which were also associated with multiple infection by this virus.

Viral load was evaluated by real-time PCR (RT-PCR) and the data was correlated by follow-up lasting two years for determining the dynamics of the single and multiple infection patterns found in Colombia for the six HR-HPV types having the greatest prevalence in the Colombian population.

Due to the foregoing, HR-HPV-16, -18, -31, -33, -45 and -58 types were quantified as was a constitutive gene (HMBS) in cervical tissue samples taken from 219 females who were seen during a minimum of 4 visits (6 month intervals: $\pm$ 3 months).

Type-specific HPV quantification was calculated from the number of absolute copies present in the sample as well as normalised ones according to the constitutive gene for obtaining the number of viral copies per cell. Associations with the sociodemographic and clinical data compiled for this group of women was also evaluated according to the viral type present in the sample.

It was found that low HPV-31 viral load values were associated with this type's long-lasting persistence. Low HPV-16 absolute load values were indicators of a negative prognosis for change ***regarding colposcopic*** assessment (the result from the beginning of the cohort compared to the result during the last follow-up).

The results for HPV-18 and -31 types revealed less probability of the infection becoming naturally resolved during the time being evaluated. HPV-16 and -58 infection in females who did not come from Bogotá had a greater probability of the infection becoming cleared.

Analysis of viral load dynamics for each of the 6 HR-HPV types evaluated provided information about the factors influencing load reduction, such as not living in Bogotá for HPV-16, -18 and -45 types, having had more sexual companions concerning HPV-18 and -45 and concomitant sexually-transmitted diseases other than HIV for HPV-45.

Using any type of family planning method was related to increased normalised viral load for HPV-16-infected women as was using hormonal methods for HPV-18 and -31.

The viral load of white women became considerably increased on becoming infected with HPV-18 or -58. Advanced age was associated with greater viral load in HPV-31-infected women. Multiple infection and having a background of abortions increased viral load regarding HPV-45 infection.

It was shown that differing risk factors were involved in modulating viral load and that this could interfere in different ways regarding each type evaluated here; however, all displayed the same tendency when modulating multiple viral types, either all increasing or all decreasing their load.

This was the first study in Colombia involving the molecular determination of HPV and identification of type-specific frequency in several parts of Colombia involving socio-demographic characteristics and different ethnic populations and establishing the association of viral load with possible risk factors. The results highlighted the importance of characterising HPV infection in ethnically diverse populations for broadening and improving CC promotion and prevention programme scope. These results have provided information about pre-vaccination HPV distribution, thereby contributing towards following-up vaccination plans for Colombian populations and supplying knowledge about HPV epidemiology in developing countries.

ESTADO DEL CONOCIMIENTO

CÁNCER DE CUELLO UTERINO

El cuello o cérvix uterino es la porción fibromuscular inferior del útero que se proyecta dentro de la vagina. El tercio inferior del cuello del útero (exocervix, endocervix) está compuesto por un epitelio escamoso estratificado no queratinizante extendido hacia el borde del mismo y por epitelio cilíndrico que confluyen en la unión escamoso-cilíndrica. El punto medio entre el exo y endocervix, es el sitio de continuos cambios metaplásicos mejor conocido como zona de transformación celular (1).

El cáncer de cuello uterino, se considera como un problema importante de salud pública en países en vía de desarrollo, los cuales presentan altos índices de mortalidad (2). Según la Organización Mundial de la Salud y la Sociedad Americana De Cáncer, el cáncer cervicouterino (CaCu) es la segunda mayor causa de mortalidad femenina a nivel mundial, donde la más alta tasa de incidencia se encuentra en el sur y centro de América, el Caribe, sub África y el sureste de Asia (3) (ver figura 1).

La prevalencia de los VPH-AR oncogénicos tipo 16, 18, 31, 33, 35, 45, 51, 52, 58, y 59 es mayor en África y América Latina; siendo VPH-16 el más frecuente en el mundo, excepto en Indonesia y Argelia donde VPH 18 es el más común. Para el tipo VPH 45, la mayor frecuencia se da en África Occidental y las prevalencias de los tipos VPH-33, -39 y -59 se concentran en Centro y Sur América (4). La incidencia a nivel mundial es variable de acuerdo a la comunidad afectada, hay regiones con baja incidencia como Israel (3.8 por 100.000) y regiones con alto número de casos como Perú (54.6 por 100.000) (5). Actualmente, el cáncer cervical afecta principalmente mujeres entre 40 y 50 años, con una incidencia global de 400 mil casos nuevos diagnosticados cada año. La incidencia, prevalencia y mortalidad han disminuido en la mayoría de países desarrollados, influenciados por el compromiso de los gobiernos con esta patología, donde las tamizaciones son obligatorias, gratuitas y efectivas. En nuestro país se presentan entre 24 y 48 casos por 100.000 habitantes (6, 7) y en los últimos años esta incidencia no ha disminuido (8).

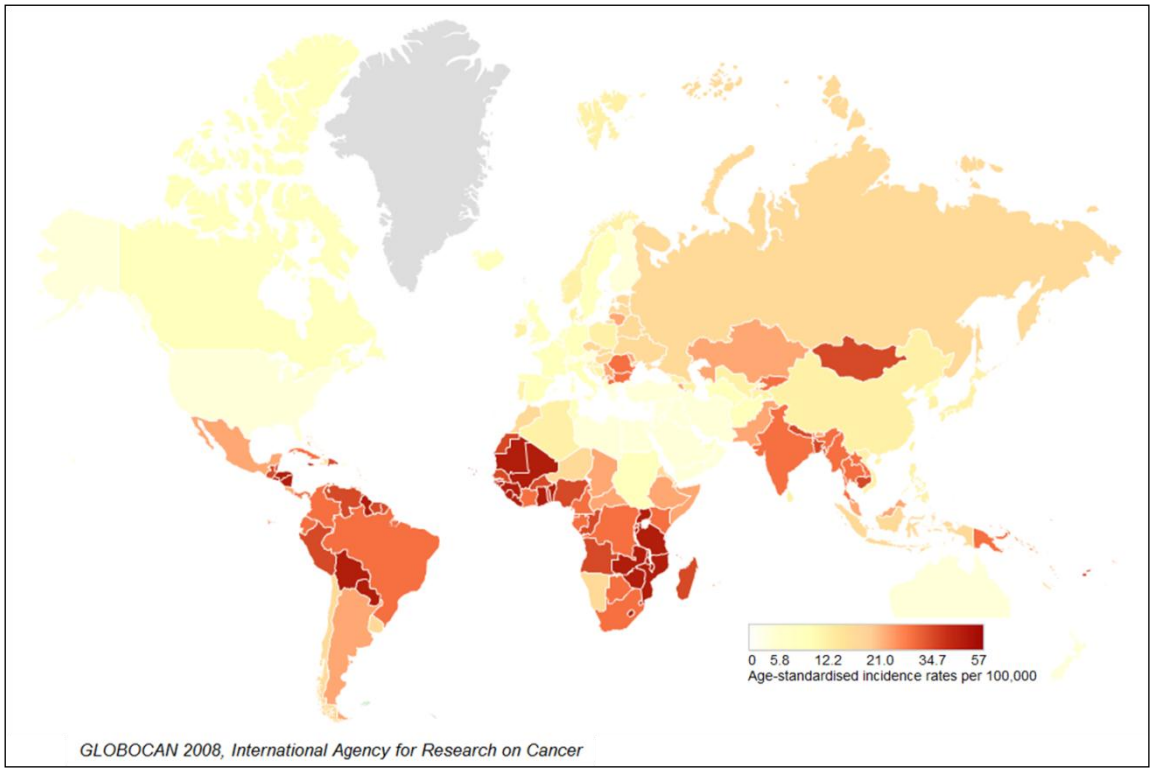

Figura 1. Tazas de incidencia de cáncer cervical, ajustado por edad. Fuente: (8).

Algunos estudios han demostrado un pico de infección antes de los 25 años, luego la prevalencia decrece rápidamente debido a que la mayoría de las infecciones son transitorias, encontrándose un segundo pico de incidencia de infección en mujeres mayores de edad, donde el riesgo de cáncer cervical aumenta (9).

Factores de riesgo para la infección

Está confirmado que el riesgo de contraer VPH está dado por contacto sexual, se habla de patrones asociados como la promiscuidad (10) e inicio de relaciones a temprana edad (11), sin embargo, se ha discutido que otros factores tales como la edad (12), el uso de anticonceptivos orales, la mala alimentación, el consumo de cigarrillo y la predisposición genética, influyen en el desarrollo de las lesiones (Revisado en (13, 14).

Del 70% de mujeres que adquieren la infección, la mayoría de las infecciones son transitorias y solo cerca del 25% desarrollan una lesión intraepitelial de bajo grado (LSIL siglas en inglés de: Low squamous intraepithelial lesion), de las cuales un 20 al 40% progresarán a lesiones intraepiteliales de alto grado (HSIL siglas en inglés de High squamous intraepithelial lesion) de acuerdo a el sistema el Bethesda (15). Lo anterior

indica que del total de mujeres que en alguna ocasión adquieren VPH, solo el 5 o 10% de ellas desarrollarán una HSIL, mientras que cerca del 90% de las mujeres infectadas no mostrarán evidencia alguna del tipo viral adquirido después de 12 a 36 meses (16).

Diagnóstico y tratamiento

La detección temprana y el tratamiento oportuno del VPH en lesiones precancerosas pueden prevenir la progresión a cáncer. Los métodos principales de diagnóstico han sido la citología (Papanicolaou) y el estudio histológico, en los cuales se buscan cambios en las células de la zona de transformación del cérvix. La citología clasifica los hallazgos en: ASCUS (células escamosas atípicas de importancia no determinada), LSIL (lesiones escamosas intraepiteliales de bajo-grado), HSIL (lesiones escamosas intraepiteliales de alto-grado), y carcinoma (adenocarcinoma o carcinoma invasivo de células glandulares (15). En nuestro país, las pacientes con resultados de Papanicolaou anormales que no tienen una lesión cervical seria son evaluadas por colposcopia, y en este examen se realiza una toma de biopsia si el médico así lo considera.

Con respecto a los tratamientos, existen guías específicas de procedimiento para cada país, sin embargo, al encontrarse tejido en crecimiento anormal, generalmente se procede a realizar una cauterización o conización de la zona afectada, dependiendo del compromiso del tejido. La conización es el procedimiento en el cual el médico extrae el tejido cervical cortándolo en forma de cono, para lograr abarcar la mayor parte de tejido basal deteriorado. La escisión por medio del asa electro quirúrgica es buen tratamiento para las lesiones escamosas no invasivas; el cáncer que comienza a invadir se trata con histerectomía o con radioterapia de alta energía, con el objetivo de destruir células malignas en el cérvix, tejidos paracervicales y nodos linfáticos regionales; el cáncer localmente avanzado es tratado con radioterapia dirigida al tumor y sitios de esparcimiento (1, 17, 18).

Prevención

Cerca del 80% de los casos de cáncer cervical puede ser prevenido si se realiza la detección temprana. No obstante, muchos países de bajos recursos no tienen las herramientas ni la infraestructura de salud pública para apoyar estos análisis.

Los requisitos de un programa organizado (según criterios de la OMS) los cumplen los países del norte de Europa, en donde es clara la relación entre el nivel de organización y la reducción de la mortalidad. Sin embargo, en los países desarrollados, se ha reportado un importante aumento de adenocarcinomas cervicales, condición que ha sido asociada principalmente con la infección por VPH-18 y la cual es más compleja de tamizar en las pruebas de diagnostico citológicas, por lo que se ha promovido la identificación molecular y respectiva cuantificación de los tipos virales de la especie A7, a la que éste pertenece (19).

Otra manera de prevenir el desarrollo de lesiones cervicales es mediante vacunas sintéticas profilácticas como las desarrolladas por Merck y GlaxoSmithKline (Gardasil y Cervarix, respectivamente) que han mostrado resultados satisfactorios y muy prometedores. Estas vacunas se construyeron basadas en la integración de las partículas similares a virus (VLPs, del inglés virus-like particles) de diferentes tipos de VPH. La primera es una vacuna tetravalente diseñada a partir de VLP-L1 de los tipos 6, 11 (los cuales causan del 75-90% de verrugas genitales externas), 16 y 18 (quienes causan el 70% de cáncer cervical) y la segunda es bivalente compuesta de los tipos 16 y 18. Ambas vacunas necesitan ser mantenidas en cadena de frio y son administradas intramuscularmente en el área deltoides. La estrategia que se empleó involucra la producción de las VLPs de cada tipo por separado y durante la formulación final los diferentes tipos son mezclados; las dos vacunas integran en su componente principal VLPs, pero cada una difiere en aspectos de formulación (20).

Las vacunas profilácticas se encuentran en estudios clínicos de Fase IV, por lo tanto no se ha podido determinar el tiempo de efectividad, ni la posible inmunidad cruzada que potencialmente pudiese proteger a las personas vacunadas contra los otros tipos virales de alto riesgo. Además, estudios recientes sugieren que las diferencias biológicas y funcionales entre las llamadas variantes virales, pueden tener impacto en la etiología del cáncer (21); la distribución geográfica y el potencial oncogénico de éstas pueden proporcionar datos que ayudarán a un diseño más eficiente de vacunas y protocolos de vacunación.

VIRUS DE PAPILOMA HUMANO

El Virus de papiloma humano pertenece a la familia Papillomaviridae, una familia de virus que infectan específicamente el epitelio escamoso de más de 20 especies

diferentes de mamíferos, así como también aves y reptiles (22). De acuerdo a la literatura, se han descrito 118 tipos de VPH de los cuales se ha determinado que 15 de ellos infectan el epitelio escamoso; algunos de ellos se clasifican como tipos virales de alto o bajo riesgo (HPV-HR, -BR) y se han agrupado en géneros denominados por letras griegas, tales como alfa, beta y gama papilomavirus, entre otros (ver figura 2) (23).

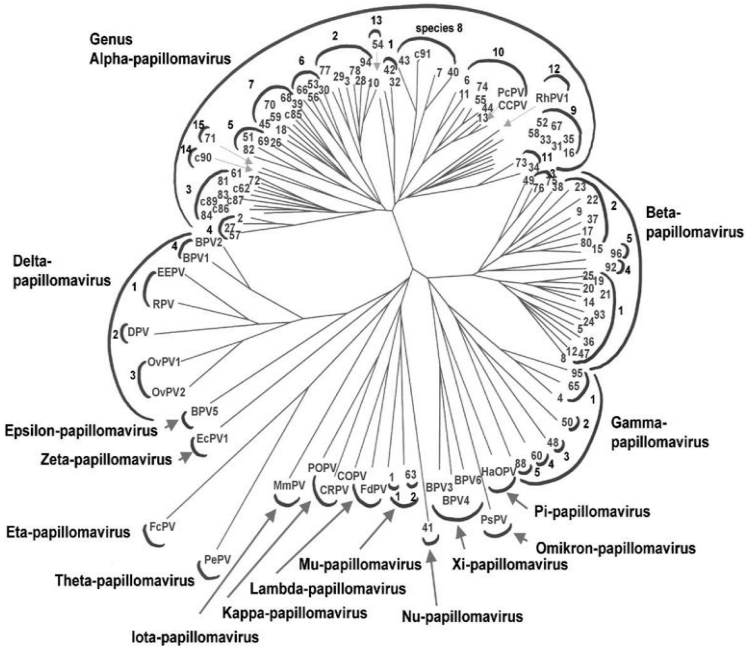

Figura 2. Clasificación filogenética de los HPV´s. Fuente: (23).

La partícula viral del papiloma humano tiene una cápside de 72 capsómeros (60 hexámeros y 12 pentámeros), con un diámetro aproximado de 55 nm que contiene al genoma viral. Los capsómeros están hechos de dos proteínas estructurales: L1 en mayor proporción y L2. El genoma del VPH, consiste en una molécula de ADN circular de doble cadena de aproximadamente 8 Kb, dividida en tres regiones: la región larga de control (LCR), que no contiene marco de lectura alguno; la región de proteínas tempranas (E1 a E8) y la región de proteínas tardías (L1 y L2) (Figura 3) (24).

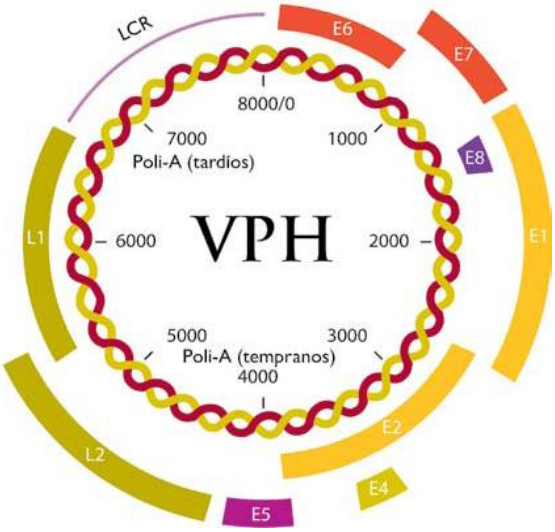

Figura 3. Organización del genoma del virus. Fuente: (1).

Los genes E codifican las proteínas reguladoras (E1, E2, E3, E4, E5, E6, E7 y E8) necesarias para los procesos de replicación, trascripción y cito transformación. E1 y E2 juegan un papel importante en la replicación del genoma viral, iniciando E1 la replicación de ADN, mientras que E2 es un factor de transcripción que interactúa con los motivos $ACCN_6GGT$ en el LCR viral. Las proteínas E2 de VPH-AR tienen la capacidad de actuar como activadores transcripcionales, además, se asocian con la helicasa de ADN viral E1. Esta interacción es necesaria para el reconocimiento eficiente del origen y replicación del genoma viral (25)

En infección de células basales y parabasales, los bajos niveles de proteína E2 activan la transcripción de E6 y E7 y, por lo tanto, la proliferación celular, además, E2 contribuye a la partición del genoma viral en las células hijas y los altos niveles reprimen la transcripción de E6 y E7 (Ver Figura 4). E2 es un candidato para el desarrollo de una vacuna debido a que es requerida para la formación de la partícula viral de papiloma, y a que está involucrada en todos los estados del ciclo y se expresa en lesiones premalignas al igual que en algunos cánceres (26).

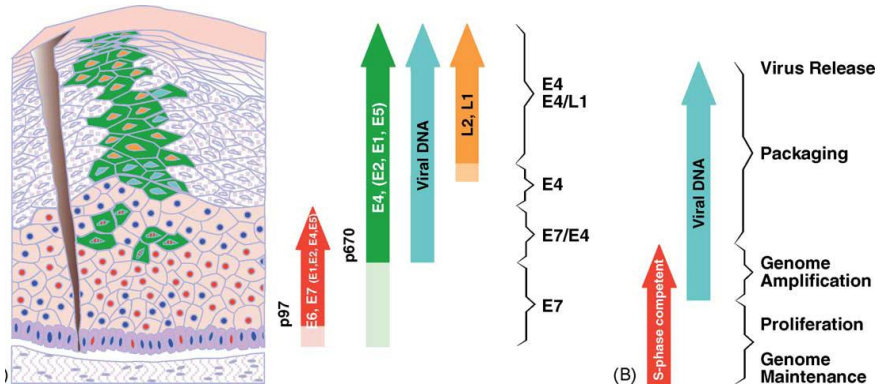

Figura 4. Organización del ciclo de vida durante la infección productiva. Fuente: (27).

La proteína E4 codifica la proteína tardía NS, actuando al final del ciclo celular, ya que tiene la capacidad de modificar el citoesqueleto para facilitar la liberación de los viriones. La denominada E5 actúa como proteína transformante, interactuando con los factores de crecimiento celular (27). Las proteínas E6 son pequeñas (de aproximadamente 150 aminoácidos) y contiene dos dominios que consisten de motivos CXXC apareados, cada uno de los cuales está relacionado con carboxi-terminal de la proteína E7. La proteína E7 es una fosfoproteína ácida unida a zinc que es detectada en el citoplasma y en el núcleo de las células transformadas por VPH, esta proteína interactúa con la proteína supresora de tumor de retinoblastoma (pRB) y daña su función como regulador del crecimiento celular (1).

Los genes L codifican las proteínas mayores de la cápside viral (L1 y L2), éstas son las responsables del ensamble de nuevos viriones en las capas cutáneas superiores. La proteína principal de la cápside es L1, que comprende más del 90% de las proteínas del virión y es el principal componente de la superficie del virus; L1 pesa 56 kDa y tiene la característica de asociarse formando partículas similares al virus. La proteína está implicada en la invasión del virus a las células epiteliales (27). La unión entre estas proteínas tiene consecuencias importantes para el empaquetamiento de ADN viral. L1 interactúa con L2 a través de un dominio hidrofóbico en una proximidad cercana al carboxi-terminal de L2. L2 despliega un dominio de unión a ADN de VPH y también un epítope expuesto en la superficie, que media específicamente en el reconocimiento inmune (28).

Existen más de 100 tipos de VPH, clasificados mediante la comparación de las secuencias de nucleótidos de regiones de replicación temprana (E) y tardía (L). La identidad de L1 debe sobrepasar el 90%, para que los virus pertenezcan al mismo tipo

(23). Estos virus generan diversas enfermedades, así: verrugas plantares (tipos 1, 2, 4, 64), verrugas comunes (tipos 1, 2, 4, 26, 27, 29, 41, 57, 65, 77), lesiones verrugosas de los labios superior e inferior, papilomas conjuntivales (tipos 6 y 11), condiloma acuminado (tipos 6, 11, 30, 42, 42, 44, 45, 51), LEI-AG (tipos 6, 11, 16, 18, 31, 33, 34, 35, 39, 42, 44, 45, 51, 52, 56, 58, 66), cáncer cervical (tipos 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, 68, 70), papilomas laríngeos y enfermedad respiratoria crónica en neonatos (tipos 6 y 11) (29, 30). Los diferentes genotipos del VPH pueden clasificarse como de alto riesgo (tipos 16 y 18), de riesgo intermedio (VPH tipos 31, 33, 35, 39, 45, 51, 52, 82) o de bajo riesgo (VPH tipos 6 y 11, 26, 40, 42, 43, 44, 53, 54, 55, 62, 66) según su capacidad de inducir cambios histológicos de carcinoma escamoso. Mientras el genotipo 16 se asocia con mayor frecuencia a carcinomas escamocelulares, el genotipo 18 a adenocarcinomas (31).

Métodos de identificación

La técnica de Papanicolaou, que se introdujo hace más de 60 años, ha demostrado ser una poderosa herramienta en la detección de lesiones precursoras de cáncer de cuello uterino. Como prueba de tamización, la citología cervical es eficaz para la detección de cambios neoplásicos cervicales. En un metanálisis se encontraron datos que indicaron valores de sensibilidad para la citología convencional de 47% con un reporte de falsos negativos muy variables que van del 1.5% al 55% (32). De manera similar, otros análisis retrospectivos en pacientes que desarrollan cáncer de cérvix a pesar de haber estado sometidas a tamización, se encontró que del 5% al 15% tenían un diagnóstico "falso negativo" y fueron casos que correspondían a ASCUS, LEI-BG y LEI-AG (33, 34).

Las limitaciones de la técnica de Papanicolaou son debidas a la proporción de muestras no valorables o poco significativas y a una sensibilidad limitada por varios factores. Se estima que aproximadamente el 10% del error del diagnóstico se atribuye al laboratorio y 16% al muestreo y a la preparación de la muestra, lo que lleva a obtener material poco representativo o significativo (35). Además, no todas las células recogidas del cuello uterino quedan adheridas a la lámina porque parte de ellas pueden quedar adheridas al citocepillo, por lo tanto, las células con anomalías representativas de una lesión pueden no ser encontradas en la lámina (36). Otras causas señaladas son la dificultad de

interpretación citológica propiamente dicha, al ser poco reproducible, y requerir mucho trabajo; adicionalmente, a la fecha, no se ha podido generar un programa automatizado para obtener un rápido y mejor procesamiento (37).

Otras técnicas muy utilizadas para establecer diagnóstico son las que involucran inmunofluorescencia e inmunoquímica, sin embargo, se enfrentan al problema de la baja sensibilidad, especificidad y reproducibilidad. El uso de anticuerpos policlonales anti-proteínas estructurales tardías L1 y L2 puede confirmar la presencia de cambios morfológicos inducidos por VPH, pero no ha superado la sensibilidad que muestra el diagnóstico de ADN de VPH (38). En un estudio previo, utilizando la técnica de inmunohistoquímica, se demostró que el porcentaje de detección de L1 en lesiones premaliganas de alto grado fue menor que en lesiones de bajo grado, siendo 50% y 69% respectivamente, esto es debido a que a medida que el proceso neoplásico aumenta en severidad, la transcripción de L1 y L2 tiende a desaparecer, por lo que en las LEI-AG y en el cáncer invasivo es poco probable detectar dichas proteínas (39).

Los análisis por metodologías de captura antigénica como el ELISA han ofrecido buenas perspectivas, pero aún no se ha desarrollado un método de tamización confiable (40-42). Existe evidencia que un estudio serológico puede ser usado como una prueba de tamización complementario a la citología (43). Los ensayos son de fácil manipulación, no requieren equipo especial y podrían ser realizados por muchos laboratorios clínicos alrededor del mundo. Como marcadores serológicos en cáncer cervical se ha utilizado el antígeno celular de carcinoma escamoso (SCCA), derivado de las células cervicales cancerosas, y el antígeno carcinoembrionario (CEA); sin embargo, sus aplicaciones clínicas son criticadas debido a que los valores estándar de positividad son demasiado amplios (valores tan bajos como 34% y 18%, respectivamente) y además presentan cambios inexplicables en los estados tempranos del cáncer cervical y no están relacionados con el estado clínico (44).

En la actualidad, se han desarrollado técnicas moleculares con el fin de detectar e identificar el ADN viral; una de las más empleadas es la Reacción en cadena de la polimerasa (PCR siglas en inglés de: Polymerase chain reaction) (45, 46). Ésta es una técnica muy sensible y especifica (47), permite identificar la presencia de ADN viral, incluso cuando hay bajas copias en la muestra. Sin embargo, presenta limitantes relacionadas con la selección de los sets de cebadores adecuados y la estandarización de

los protocolos. Dentro de los juegos de cebadores que se han desarrollado para la identificación de VPH, se encuentran aquellos que serán utilizados en el presente proyecto y se denominan GP5+/6+, el cual permite detectar baja cantidad de ADN de VPH (48) y MY09/11 diseñado para detectar infección en la existencia de múltiples tipos virales (49).

En la actualidad existen 4 pruebas avaladas por la FDA para la detección de la infección por VPH. Dos de ellas se basan en ensayos de amplificación de señal como lo son Digene HPV test y Cervista ® HPV HR. La primera de es una prueba que utiliza la tecnología de Captura Hibrida ®, identificando la amplificación de señales no radioactivas, generadas tras la hibridación en solución de blancos de ADN de VPH y sondas de RNA marcadas; ésta permite hacer la distinción entre infecciones relacionadas con tipos de VPH de alto y bajo riesgo, pero no genotipifica (50, 51).

El ensayo Cervista ® HPV HR, utiliza un método de amplificación de señales para la detección de ácidos nucléicos del virus a través de sondas específicas, detecta la presencia de 14 genotipos de VPH de alto riesgo y una modificación de este ensayo permite hacer la distinción exclusiva para los tipos VPH-16 y -18 (50, 51).

Una tercera prueba avalada por la FDA es cobas HPV Test ®, que detecta ADN de 14 tipos de VPH por medio de amplificación de ácidos nucleicos del virus a través de la técnica de Reacción en Cadena de la Polimerasa en Tiempo Real, utilizando un sistema automatizado, con ésta se logra discriminar los tipos VPH-16 y -18, detecta adicionalmente otros 12 tipos de VPH. La última prueba es APTIMA® HPV Assay, la cual se basa en la detección de RNA de los oncogenes virales que codifican para las proteínas E6 y E7 (50, 51).

arias metodologías han intentado combinar la PCR con el Western Blot, procurando aumentar la sensibilidad de la prueba. Uno de los más usados hasta el momento, es un par de cebadores PGMY L1, cuya sensibilidad se calculó entre el 91 al 100% al ser analizado en diferentes laboratorios (52).

Tabla 1. Pruebas de identificación de ADN de VPH más utilizadas.

Método de detección de ADN de VPH	Sensibilidad %	Especificidad%	Ventajas	Desventajas	Referencias

GP5+/6+	51	100	Alta sensibilidad, especialmente con muestras que contienen bajas copias virales. Gracias a su bajo costo y fácil implementación han sido altamente usados en estudios clínicos y epidemiológicos.	Puede ser poco eficaz en la amplificación de algunos tipos de VPH-AR tales como VPH-53 y -61. No recomendado para tipificación.	(48, 53)
MY09/11	72.2	100	Detección de más de 25 tipos virales simultáneamente. Alta sensibilidad en la detección de diferentes genotipos. Gracias a su bajo costo y fácil implementación han sido altamente usados en estudios clínicos y epidemiológicos.	Fragmentos de PCR, relativamente largos. Existe la posibilidad de la pérdida del blanco de amplificación, especialmente en muestras que tienen poco producto de ADN tales como las fijadas en formalina, embebidas en parafina o con alto porcentaje de integración. No apropiado para tipificación.	(49)
PGMY-LB	95.6	----	Combina ensayos de PCR con marcaje tipo específico de sondas oligonucleotídicas (radio-marcadas) para incrementar la sensibilidad y reproducibilidad de la detección.	Es más sensible al efecto de inhibidores en las muestras.	(54)
SPF10-LiPA25	----	----	Sistema de detección y genotipificación capaz de amplificar hasta 43 diferentes tipos; identifica simultáneamente hasta 25 tipos.	Puede tener problemas de sensibilidad en muestras con más de un tipo viral, debido a la competencia de tipos de VPH según su concentración en la muestra.	(55, 56)
Amplicor HPV test	96.4	100	Detecta 13 tipos de VPH-AR en simultánea, además asegura la presencia del gen de la β-globina humana como control positivo.	Hibridación cruzada entre cebadores principalmente con VPH-56 y requiere profesionales expertos para la implementación de la prueba. Requiere de más tiempo que las otras pruebas para la obtención de resultados.	(57)
LINEAR ARRAY HPV	89.1	46.5	Detecta 37 tipos de VPH individualmente,	Baja sensibilidad (identificación de mujeres	(58)

genotyping test			incluyendo los 14 carcinogénicos principales. Utiliza un control interno (β-globina humana).	con la enfermedad). Las muestras deben mantenerse en medio especial (STM).	
PapilloCheck HPV-Screening Test	----	----	Detecta y tipifica 25 tipos de VPH en simultánea: 15 VPH-AR, 2 tipos de VPH de riesgo probable y 8 VPH-BR.	Puede reportar discrepancias en la detección al compararlo con otros métodos de detección múltiple. Se ha reportado una importante detección de falsos positivos. La detección se dirige a E1, gen susceptible a deleciones parciales comparado con la región L1.	(59)

PREGUNTAS DE INVESTIGACIÓN

Componente de corte Transversal:

Cuál es la prevalencia de infecciones únicas y múltiples para poblaciones socio-demográficamente heterogéneas?

Cuál es la distribución tipo-específica de VPH en nuestra población?

Existen factores de riesgo asociados con las infecciones únicas y múltiples?

Componente Longitudinal:

Existe correlación entre los niveles de carga viral de los diferentes genotipos a lo largo del tiempo?

OBJETIVOS

Para abordar los componententes descritos, se plantearon los siguientes Objetivos Generales:

Caracterizar los patrones de infección por virus de papiloma humano en un grupo de mujeres en riesgo de contagio.

Determinar la dinámica de los patrones de infecciones únicas y múltiples para seis tipos del virus de papiloma humano de alto riesgo.

El cumplimiento de estos objetivos generales fue enmarcado en la ejecución de los siguientes

Objetivos específicos:

Describir las características socio-demográficas y clínicas de las mujeres que participan en este estudio.

Evaluar la asociación entre infección única o múltiple con factores de riesgo para infección con VPH.

Determinar la prevalencia de VPH en el grupo de mujeres participantes en el estudio.

Determinar las prevalencias tipo-específicas para seis tipos de VPH-AR en grupos de mujeres provenientes de diferentes regiones del país.

Establecer asociaciones entre las infecciones únicas y múltiples de VPH y los factores de riesgo evaluados.

Cuantificar en diferentes puntos de seguimiento el ADN genómico y el número de copias virales presentes en muestras cervicales de un grupo de mujeres infectadas con VPH.

Describir la dinámica de los valores de carga viral de VPH de Alto riesgo en mujeres con infección única o múltiple.

Comparar los valores de carga viral a lo largo de los seguimientos dependiendo de la presencia o no de coinfección.

INTRODUCCIÓN A LOS CAPÍTULOS

El cáncer de cuello uterino (CCU) es un grave problema de salud pública, siendo la neoplasia más común entre las mujeres, especialmente en aquellas que habitan países en vías de desarrollo. En Colombia, se reporta una incidencia de este tipo de cáncer de 18,7 por cada 100.000 mujeres al año (60), la cual no disminuye desde el registro del 2007 (61), lo cual podría estar relacionado con los cambios presentados en las conductas sexuales, tales como aumento en la promiscuidad e inicio temprano de la vida sexual.

Un factor necesario pero no suficiente para que se desarrolle este tipo de cáncer es la infección con el VPH (62); se han reportado más de 200 tipos de VPH, de los cuales 85 tienen caracterizado su genoma. Según la asociación que presentan con el cáncer cervical y las lesiones que provocan, han sido clasificados en virus de bajo (VPH-BR) y alto riesgo (VPH-AR) (23). Los virus de alto riesgo más importantes son VPH-16 y VPH-18, con una prevalencia mundial de 58,9% y de 15,0%, respectivamente (61).

La principal herramienta para el diagnóstico de CCU es el análisis citológico del tejido cervical a partir de la tinción de Papanicolaou, sin embargo, a pesar de su bajo costo y elevada especificidad (95-98%) (63), esta técnica presenta una limitada sensibilidad para la detección de lesiones precancerosas (41-73%) (64), lo que se ha intentado solucionar realizando citologías siguiendo el esquema nacional 1: 1: 3 (17).

En los últimos años, se ha trabajado en el desarrollo de métodos complementarios para la detección de mujeres en riesgo de desarrollar CCU; los métodos más utilizados se basan en la detección directa de ADN viral de VPH, mediante el empleo de técnicas como la Reacción en Cadena de la Polimerasa (PCR), ya que ésta provee una mayor sensibilidad y especificidad; adicionalmente, se han desarrollado técnicas que además de permitir la identificación viral, determinen el número de copias virales presente en la muestra, tales como la PCR en tiempo real.

La cuantificación del virus de VPH es un factor que ha sido considerado importante desde el punto de vista clínico, ya que se ha demostrado que la persistencia de la

infección y el mantenimiento elevado de la carga viral, contribuyen a la progresión de lesiones de bajo grado a cáncer cervical (65, 66).

Uno de los principales intereses científicos en la investigación en VPH es la determinación de persistencia o eliminación del virus por parte del organismo infectado. Ha sido establecido que una infección persistente por VPH-AR, constituye uno de los factores de riesgo más importantes, sin embargo, es necesario la realización de más estudios, con la finalidad de establecer las características de la regresión y/o progresión de enfermedades cervicales.

Para lograr discernir cuáles son los factores intrínsecos, tanto del virus como del hospedero, que permiten la regresión o el desenlace de la infección, se ha realizado la identificación de la carga viral para VPH en muestras de mujeres con varios grados de lesión (67, 68), con varios tipos virales (69-71), y con muestras tomadas en diferentes fluidos (72, 73), entre otros.

La cuantificación del ADN viral ha permitido relacionar el aumento en el número de copias virales con el aumento en el grado lesión, principalmente para los tipos VPH-16 y -18 (74, 75). Se ha sugerido que en grados avanzados de lesiones cervicales, el tipo VPH-16 se encuentra con mayor número de copias, principalmente integrado al genoma del hospedero (76), mientras que para VPH-18, la correlación entre el aumento en su carga viral y el aumento de la gravedad de la lesión, no ha sido claramente establecida (77); sin embargo, se reporta con mayor frecuencia el VPH-18 ligado a lesiones más avanzadas y en menor carga viral comparado con VPH-16 (78).

Se han llevado a cabo estudios longitudinales alrededor del mundo, con el objetivo de establecer si existen diferencias entre las cargas virales de varios tipos de VPH, prestando mayor interés a la cinética de la carga viral para VPH-16. Éste es el caso de dos estudios que demostraron que sólo después de dos seguimientos (cada seis meses), se pueden observar fluctuaciones en la carga viral para VPH-16, y que en el caso de encontrar una gran disminución de la misma en al menos 1 unidad en la escala logarítmica, habría menos riesgo de desarrollar algún tipo de lesión cervical (79, 80). Sin embargo, en ambos estudios se argumenta que hace falta tener tamaños de muestra mayores (en uno realizaron seguimiento a 39 mujeres y en el otro a 50) y análisis de otros tipos virales.

Con respecto a la cuantificación de varios tipos de VPH-AR, en un estudio llevado a cabo en mujeres irlandesas, se reportó una mayor carga viral de VPH-31 al compararlo con la cuantificación para VPH-16 y -18, pero no se encontraron diferencias estadísticamente significativas entre la variación de la carga viral de VPH-16 y la presencia de infecciones únicas o múltiples. Sin embargo, los autores argumentan que el tamaño de la muestra fue bajo (39 mujeres) y que sólo se tomó un seguimiento al año de la primera toma; por lo tanto, la conclusión fue similar en cuanto a la falta de estudios con tamaños de muestra mayores que permitan establecer asociaciones entre carga viral de VPH-AR y el desarrollo de lesión, o que identifiquen el rol de las coinfecciones por VPH en la cinética de carga viral para los tipos más prevalentes (81).

Existen algunos estudios de cohorte donde se analiza la cuantificación viral de VPH, no obstante, no se han encontrado diferencias entre infecciones únicas o múltiples y el desarrollo de lesiones (82). En un estudio de cohorte prospectiva llevado a cabo en Turku-Tailandia, se demostró que existe una tendencia al aumento de los tipos virales de alto riesgo pertenecientes a la especie 9 (VPH-16, VPH-31, VPH-33, VPH-58) y cierta disminución de aquellos pertenecientes a la especie 7 (VPH-18 y VPH45) a través del tiempo, siendo estos hallazgos más evidentes en el mes 36, luego de realizar seguimientos cada 6 meses. Además, dentro de los seguimientos, las mayores prevalencias fueron de VPH-16, seguido de las infecciones múltiples (83).

El interés en infecciones múltiples había disminuido, ya que se había propuesto la clonalidad del cáncer cervical (19); sin embargo, se ha sugerido que las infecciones múltiples favorecen las condiciones para que un solo tipo viral logre llevar a cabo su ciclo replicativo de forma eficiente y colonice el tejido cervical (84). A pesar de esto, la detección simultánea de varios tipos virales y su respectiva carga viral en muestras con lesiones, ha sido poco evaluada; se ha logrado determinar que en coinfecciones, las cargas de VPH-16 y -18 se reducen, presentándose una mayor reducción al estar en presencia de tipos no relacionados filogenéticamente (85).

En Colombia, se realizó un estudio longitudinal en el cual se logró determinar que la carga viral es el principal determinante de persistencia, y para el tipo VPH-16, ésta representa el mayor riesgo en el desarrollo de lesiones de alto grado (86), a pesar de ser muy debatido el papel que desempeña la carga viral, como marcador predictivo de lesiones cervicales de mal pronóstico (87, 88).

Es importante resaltar que el presente estudio fue diseñado con dos enfoques. El primero, buscó identificar las principales características de la infección por VPH en un grupo de mujeres en riesgo de contagio, determinando prevalencias de infecciones únicas y múltiples, prevalencias tipo-específicas y se evidenciaron las variables sociodemográficas asociadas con el riesgo a la infección. El segundo enfoque, identificó los parámetros de depuración viral, persistencia y reinfección de los seis tipos de VPH-AR más frecuentes en Colombia, y determinó la dinámica de la carga viral VPH tipo-específica y la relación con factores sociodemográficos que pudiesen modularla.

Para abordar el primer enfoque, se realizó un estudio transversal que se describe en los capítulos 1 al 3; en estos capítulos, se corrobora que en Colombia, la distribución de los tipos de VPH es diferencial, tal y como se observa en poblaciones heterogéneas alrededor del mundo. En el primer capítulo de este documento, se describen las características de las infecciones por VPH de la población colombiana, con respecto a las prevalencias virales y a las asociaciones de éstas con distintos factores de riesgo (características socio-demográficos y de comportamiento sexual).

En el segundo capítulo, una vez establecidas las características de la infección por los 6 tipos de VPH-AR incluidos y los 2 de VPH-BR en la población analizada, se evaluaron los patrones de infección única y múltiple. Los resultados mostraron que éstos no obedecen a una distribución al azar y se logró identificar una asociación entre un mayor número de tipos virales presente en las infecciones múltiples, con las variables sociodemográficas.

En los capítulos 3 y 4 se presentan las características operativas y la aplicación de la técnica molecular empleada en el estudio. Los resultados mostraron que el uso de más de un juego de cebadores para la identificación de VPH da robustez e incrementa la sensibilidad en los estudios epidemiológicos. En el cuarto capítulo, se implementó un juego adicional de cebadores, pU1M/2R, el cual amplifica un fragmento presente en la región codificante para las proteínas virales de mayor relevancia en el secuestro de la maquinaria celular. Adicionalmente se determinaron las características operativas para la detección de la infección, para la cual se tomó como prueba de referencia imperfecta Captura Hibrida II (CHII), que para ese entonces era la única prueba avalada por la Food and Drug Administration (FDA) para detectar la infección por VPH.

Los dos capítulos finales del documento, contienen el desarrollo del segundo enfoque de esta tesis, el cual exlica la necesidad que existe en determinar la dinámica de los patrones de infecciones únicas y múltiples para seis tipos de VPH-AR. En el quinto capítulo se muestran los datos de persistencia, depuración y re-infección para cada uno de los seis tipos virales evaluados, determinando también la relación de la infección tipo específica, la carga viral y otros factores de riesgo.

Finalmente, en el último capítulo se reporta el análisis a lo largo del tiempo, de las cargas virales (para los seis VPH-AR incluidos), para lo que se tuvo en cuenta el cambio de este factor, en cada uno de los períodos de seguimiento; éste capítulo final no se presenta como publicación final, ya que fue recientemente sometido a evaluación por pares a la revista “PLoS ONE”.

En el presente trabajo se logró establecer la presencia de los patrones de infección única y múltiple por VPH en una población de mujeres heterogénea como la colombiana, a partir de un estudio transversal realizado en cinco regiones diferentes del país. En una segunda fase longitudinal, se evaluó la carga viral mediante PCR en tiempo real, correlacionando los datos encontrados a través del tiempo, con la depuración del virus, la persistencia y la reinfección tipo-específica.

CAPÍTULO 1

"Frequency of human papillomavirus infection, coinfection, and association with different risk factors in Colombia"

ELSEVIER

Frequency of Human Papillomavirus Infection, Coinfection, and Association with Different Risk Factors in Colombia

MILENA CAMARGO, BSc, SARA C. SOTO-DE LEON, BSc, RICARDO SANCHEZ, MD, MSc, ANTONIO PEREZ-PRADOS, PhD, MANUEL E. PATARROYO, MD, AND MANUEL A. PATARROYO, MD, DrSc

PURPOSE: The aims of this study were to provide new insights into infection patterns of six high-risk human papillomaviruses (HR-HPV-16, -18, -31, -33, -45, and -58) and two low-risk HPV types (LR-HPV-6 and -11), their association with risk factors and coinfection.
METHODS: Cervical samples of 2110 women were tested for the presence of HPV–DNA by polymerase chain reaction. Statistical analyses were performed to determine viral-type frequencies in single and multiple infections and association between infection and different risk factors.
RESULTS: HPV-16 was the most prevalent type among the studied population, followed by HPV-31. This last viral type showed a variable distribution between the different cities evaluated. The results showed distinct type-specific distributions among regions and a high association between absence of pregnancies, cities as Girardot and Leticia, the indigenous ethnicity, and coinfection.
CONCLUSIONS: The results showed a variable distribution of HPV types according to the geographical region analyzed. In addition, data suggest that some sociodemographic-factors such as ethnicity, number of pregnancies, lifetime number of sexual partners, and geographic region were significantly associated, and our results showed little differences between single and multiple infections by HPV with regard to risk factors. Furthermore, these results provide relevant information that will allow assessing in further studies the impact that vaccination programs on these populations and the selective pressure would have on the distribution of HPV types.
Ann Epidemiol 2011;21:204–213.

KEY WORDS: Cervical Intraepithelial Neoplasia, Colombia, Epidemiology, Papillomavirus Infections, Risk Factors.

INTRODUCTION

Cervical cancer (CC) is the second most-common type of cancer among women worldwide. Human papillomavirus (HPV) is recognized today as the main causal factor of virtually all CC cases, given that high-risk (HR)-HPV DNA has been detected in approximately 99.7% of women with CC (1). In Colombia, the annual incidence of CC is estimated to be 32.9 cases/year per every 100,000 women, and HPV infections are detected in 6.2 of every 100 women per year, with women younger than the age of 20 being the most affected population (2, 3). CC is a major public health issue in Colombia because approximately 18.1 of every 100,000 women at an average age of 46.5 years die annually as a result of this disease (3).

Previous studies conducted in women from Bogotá D.C. (Colombia) found that nearly 20%–30% of all HPV-infected women are infected with more than one HPV type, which can be either phylogenetically related or unrelated and acquired simultaneously or subsequently (4). Nevertheless, in another study performed in rural populations of Colombia, with lower socioeconomic background, the authors (5) found that the prevalence of HPV could be even greater (36%).

The aim of the present study was to establish the prevalence of the HR-HPV types 16, 18, 31, 33, 45, and 58 (which are associated with ~90% of all CC worldwide), as well as the low risk (LR)-HPV types 6 and 11 (mainly involved in anogenital warts) in women inhabiting five geographical regions of Colombia and having different socioeconomic backgrounds: Chaparral, Girardot, Engativá, Leticia, and Tumaco. These five regions comprise socially heterogeneous

From the Fundación Instituto de Inmunología de Colombia, Bogotá, Colombia (M.C., S.C.S.-D.L., R.S., M.E.P., M.A.P.); Universidad del Rosario, Bogotá, Colombia (M.C., S.C.S.-D.L., M.A.P.); Universidad Nacional de Colombia, Bogotá, Colombia (R.S., M.E.P.); and Universidad Pública de Navarra, Pamplona, Spain/SADAR Association (A.P.-P.).

Address correspondence to: Manuel A. Patarroyo, MD, DrSc, Cra. 50 No. 26-20, Bogota, Colombia. Tel.: +5713244672 Ext. 141; Fax: +5714815269. E-mail: mapatarr.fidic@gmail.com.

The authors thank the Asociación Investigación Solidaria SADAR, Caja Navarra, Navarra, Spain, and the Spanish Agency for International Development Cooperation (Project 08-CAP2-0609) for financing this project.

Received June 1, 2010; accepted November 7, 2010.

1047-2797/$ - see front matter
doi:10.1016/j.annepidem.2010.11.003

Selected Abbreviations and Acronyms

CC = cervical cancer
HPV = human papillomavirus
HR-HPV = high-risk human papillomavirus
LR-HPV = low-risk human papillomavirus
PCR = polymerase chain reaction
OR = odds ratio
CI = confidence interval

female populations with high and low risks of developing CC (6), which allows establishing a correlation between cytological findings and molecular data from an epidemiological point of view by considering different demographical features and sexually related behaviors such as age, ethnicity, geographical origin, age at first intercourse, number of full-term pregnancies, and the lifetime number of sexual partners. The study also assessed the risk factors associated with HPV infection and coinfection (i.e., infection with more than one HPV viral type).

The information gathered in this study could be relevant for HPV vaccination studies, due to the selective pressure that the introduction of new HPV vaccines may have on the distribution of HPV types, and could thus provide hints about the expected success of vaccination schedules applied to these populations, and about distribution and coinfection rates of HPV in Colombia.

MATERIALS AND METHODS

Populations

Three of the regions included in this study comprised Andean territories (i.e., Colombia's west-central region): Chaparral, which is located in the department of Tolima and has approximately 8500 inhabitants as well as low population mobility patterns; Girardot, which is the second city of importance in the department of Cundinamarca after Bogotá (Colombia's capital city), with 131,354 inhabitants and with an economy largely based on tourism; and Engativá which is a locality of Bogotá, situated on the northwestern part of the city.

Leticia was the fourth population included in this study. It is the capital of the department of Amazonas and has an average population of 39,636 inhabitants. The city is located in a jungle region at the country's southernmost edge (in the Colombian, Brazilian and Peruvian triborder), and acts as a major port on the Amazon River. Finally, the fifth region was Tumaco, a port city in the southwestern department of Nariño with 170,000 inhabitants; its coasts are bathed by the Pacific Ocean and limits with Ecuador.

These regions were selected based on the assumption that they represented people from different socioeconomical backgrounds (mainly low), culturally diverse and with different access levels to health facilities. They comprised socially heterogeneous female populations that are both at high risk of developing CC, such as Amazonas and Tolima, where mortality rates per 100,000 females per year are 5.67 and 5.55, respectively, as well as at a moderate risk (Bogotá, 3.29; Cundinamarca, 3.22; and Nariño, 3.12 per 100,000 female per year) (6).

Sociodemographic Data

A total of 2110 women were enrolled in this study. These women who were asked to take part in this study attended to their CC prevention consult between April and September of 2007 at the League against cancer, Leticia-Amazonas (n = 173), Hospital San Juan Bautista, Chaparral-Tolima (n = 174), Hospital Engativá-Bogotá (n = 921), Nuevo Hospital San Rafael, Girardot-Cundinamarca (n = 334), and Hospital San Andrés, Tumaco-Nariño (n = 508). The women filled out a questionnaire regarding sociodemographic features, sexual behaviors, and risk factors before undergoing gynecological examination (Table 1). Five women between the ages of 70 and 77 years were excluded from the statistical analysis because Colombian legislation indicates that no screening by conventional cytology is required for women older than 69 years (7).

Ethical Approval

Before undergoing gynecological examination, all female subjects provided their written informed consent to provide a cervical sample for Papanicolaou testing and polymerase chain reaction (PCR) HPV-DNA detection; informed consent of women under the age of 18 was signed by a parent or guardian. All procedures performed in this study were approved and supervised by the Ethics Committee of each institution.

Collection, Processing, and Detection of Human Papillomavirus DNA in Cervical Samples by PCR Amplification

Samples of cervical epithelium were collected with a cytobrush and kept in 95% ethanol until further analysis (8). DNA from these samples was digested in lysing buffer (10 mM Tris–HCl, pH 7.9; 0.45% Nonidet P-40; 0.45% Tween 20 and 60 μg/mL of Proteinase K; Invitrogen, Camarillo, CA), first at 60°C for 1 h and then at 95°C for 10 min. Aliquots of 2.7 μL of each processed sample were then amplified by PCR with the use of the human β-globin GH20/PC04 specific primers, to evaluate DNA integrity (9).

Two different sets of generic primers for HPV annealing in the *L1* gene were used to detect HPV-DNA. We took into account studies in which the authors reported that the use of

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TABLE 1. Demographic profile of the 1810 women with positive human β-globin amplification

Variable	Regions*					
	Leticia-Amazonas (n = 139)	Chaparral- Tolima (n = 149)	Engativá-Bogotá (n = 796)	Girardot-Cundinamarca (n = 320)	Tumaco-Nariño (n = 406)	Total (n = 1810)
Age, years†	39.7 [17–68] SD = 12.0	35.7 [15–66] SD = 10.9	39.9 [17–69] SD = 12.1	41.0 [16–69] SD = 12.2	35.8 [14–62] SD = 10.3	38.8 [14–69] SD = 11.8
Ethnicity						
White	24.6	7.1	9.7	52.7	3.9	17.0
Indigenous	25.4	1.4	0.1	0.4	1.7	2.6
Mestizo	46.4	90.0	86.3	42.5	13.8	59.2
Black	3.6	1.5	3.9	4.4	80.6	21.2
Marital status						
Single	14.1	7.0	26.6	16.8	1.4	16.7
Married	22.4	36.6	26.7	24.8	9.4	22.9
Civil union	46.3	49.4	33.0	46.3	88.5	50.2
Separated	12.7	4.9	11.0	8.6	0.0	7.7
Widowed	4.5	2.1	2.7	3.5	0.7	2.5
Age at first intercourse, years						
<15	34.1	30.2	21.9	18.2	31.7	25.0
16–17	21.8	24.6	23.1	30.3	29.9	25.9
18–19	24.9	21.2	26.0	22.2	23.8	24.4
>19	19.2	24.0	29.0	29.3	14.6	24.7
Pregnancies						
None	9.2	0.9	7.9	7.4	4.7	6.7
1–2	29.0	27.1	41.9	35.6	28.1	35.7
2–3	31.3	42.4	36.1	37.8	31.6	35.4
>4	30.5	29.6	14.1	19.2	35.6	22.2
Cytological findings						
Normal	99.3	98.7	83.4	95.3	96.0	91.0
Abnormal	0.7	1.3	16.6	4.7	4.0	9.0
Lifetime number of sexual partners						
1	39.1	45.8	41.1	52.0	39.4	42.8
2–3	42.2	45.1	46.2	42.8	53.3	46.8
>3	18.7	9.1	12.7	5.2	7.3	10.4
Contraceptive method						
None	44.7	37.6	45.0	41.9	36.8	42.1
Oral contraceptives	6.8	4.5	3.2	10.9	5.3	5.4
Surgery	31.1	39.2	24.9	33.3	39.4	31.1
Condom	4.5	3.6	6.1	5.0	1.7	4.6
Intrauterine device	3.0	14.2	15.1	8.6	8.6	11.5
Injectable contraceptives	9.9	0.9	5.7	0.3	8.2	5.3
Smoking status						
Yes	90.7	4.0	86.0	88.4	7.6	89.0
No	9.3	96.0	14.0	11.6	92.4	11.0

Values are in percentages unless otherwise noted.
*The number of women reported for each region corresponds to samples having a good DNA quality (positive human β-globin).
†Mean [range].

different primers provides greater robustness for detecting infections with multiple HPV types compared with analyses that use a single consensus primer set (10). In brief, the supernatant gathered after processing samples with the lysing buffer was subjected to PCR amplification with GP5+/GP6+, which allows detecting low viral copies (9, 10), and MY09/MY11, which has greater sensitivity for detecting more than one viral type (11). Samples testing positive with either one or both generic primer sets, as assessed by visualization of PCR products in 2% agarose gels, were considered to be infected with HPV and underwent further PCR amplification with type-specific primers annealing within the E5-E6 and E7 regions of HR-HPV-16, -18, -31, -33, -45, and -58 (12, 13), as well as with a set of primers that allow the identification of LR-HPV-6/11, although this latter primer set does not allow discriminating between these two types (14). The HR-HPV types evaluated in this study have been linked with 90% of all CCs reported in Latin America and around the globe (15). Synthetic genes encoding HPV-18, -31, -45, and -58 regions and HPV-6,

-11, -16, and -33—infected samples were used as positive controls for type-specific identifications (16). Appropriate positive and negative controls were included in each assay to rule out DNA contamination.

Statistical Analysis

The population size was estimated with the consideration that 1778 individuals yield a 95% confidence interval, equal to the sample proportion plus or minus 0.02 when the estimated proportion is 0.25 (4). On the basis of a stratum of five geographical regions, the number of samples was adjusted considering a proportional allocation, where the size depends on the number of cytologies being taken in each region. For the statistical analysis, the age of the subject, age at first intercourse, lifetime number of sexual partners, and number of full-term pregnancies were treated as categorical variables and summarized by the use of percentages with their corresponding 95% confidence intervals (CIs). Differences between proportions were evaluated by applying a χ^2 and a Fisher exact test, whereas associations between categorical variables were assessed using odds ratios (ORs) with their corresponding 95% CIs. Binomial logistic regression was performed to evaluate the relationship between risk factors and infection (taken as a binomial outcome). In addition, an ordinal logistic regression was used for estimating adjusted ORs, considering the presence of any of the three infection levels (none, single, multiple) as the main outcome variable. An ordinal logistic model was performed to assess association between variables, including interaction terms between the variables of pregnancies, lifetime number of sexual partners, ethnicity and geographic region, for this purpose, we performed a stepwise model using 0.15 as p.e and 0.2 as p.r. These statistical procedures were carried out using STATA® software, setting the level of significance to 0.05.

RESULTS

A total of 2110 cervical epithelium samples were collected from women inhabiting the five different regions analyzed in this study. Two hundred and five samples (14%) were excluded from the study because of poor DNA quality, and five samples (0.2%) were excluded because they belonged to women older than 69 years of age. The remaining 1810 samples (testing positive for the β-globin gene) were analyzed with the two generic primer sets, identifying HPV-DNA (as indicated by the positive amplification with GP5+/GP6+, MY09/11, or both) in 897 samples (49.6%) and coinfection in 581 (64.8% of HPV-positive samples) of them. A total of 702 samples (38.8%) showed positive amplification with MY09/11 and 457 (25.2%) showed positive amplification with GP5+/GP6+. PCR assays with generic primers were carried out twice at different times to avoid false-positive readings attributable to sample contamination.

Cytological findings were classified according to the Bethesda system. According to this criteria, 92 Pap smears (5.1%) were missing/unsatisfactory, 1563 (86.4%) were negative for intraepithelial lesion or malignancy, and 155 (8.5%) showed cytologic abnormalities, with this later value being slightly greater than the percentages reported for other Colombian populations (17). There were atypical squamous cells of undetermined significance in 92 of the samples with cytologic abnormalities (5.1%), low-grade squamous intraepithelial lesion in 50 (2.7%), and high-grade squamous intraepithelial lesions in 13 (0.7%) of them.

The frequency of HPV-DNA detection was 61.1% for Leticia, 54.3% for Chaparral, 46.1% for Engativá, 51.6% for Girardot, and 49.0% for Tumaco. HPV-16 was the most frequently found viral type, followed in decreasing order by HPV 31, 18, 33, 45, 58, and 6/11. Whenever the LR-HPV types 6/11 were detected, coinfection with HR-HPV types was also detected. A similar distribution of HPV frequencies was observed in Engativá, Leticia, and Girardot; however, it is worth noting that the greatest frequency of coinfection was found in the latter two populations ($p = .010$; Table 2). In Chaparral and Tumaco, HPV-18 was the second most commonly encountered type of HPV, followed in decreasing order by HPV 31, 33, 45, and 58 (Figure 1).

Significant differences were observed between the proportions of HPV infecting each population when being analyzed regarding the place of origin of the females (Figure 1). HPV-16 was more common in Leticia-Amazonas ($p = .004$) and HPV-18 in Girardot-Cundinamarca and Chaparral-Tolima ($p = .001$). HPV-31, -33, and -45 were more frequently detected in Girardot-Cundinamarca and Leticia-Amazonas ($p = .000$), whereas HPV-58 was predominantly identified in Girardot-Cundinamarca, Leticia-Amazonas, and Engativá-Bogotá ($p = .002$), and HPV-6/11 in Chaparral-Tolima ($p = .000$).

When we calculated the association between any HPV infection (PCR amplification with at least one primer set)

TABLE 2. Relative frequency of single and multiple HPV infections with the types evaluated in this study, according to the different regions

Region	Without infection n (%)*	Single infection n (%)*	Multiple infections n (%)*	Total
Leticia-Amazonas	54 (38.9)	19 (13.7)	66 (47.4)	139
Chaparral-Tolima	68 (45.6)	31 (20.9)	50 (33.5)	149
Engativá-Bogotá	429 (53.8)	153 (19.3)	214 (26.9)	796
Girardot-Cundinamarca	155 (48.4)	19 (6.0)	146 (45.6)	320
Tumaco-Nariño	207 (51.0)	94 (23.2)	105 (25.8)	406
Total	913 (50.4)	316 (20.2)	581 (29.4)	1,810

*Percentages are calculated by rows.

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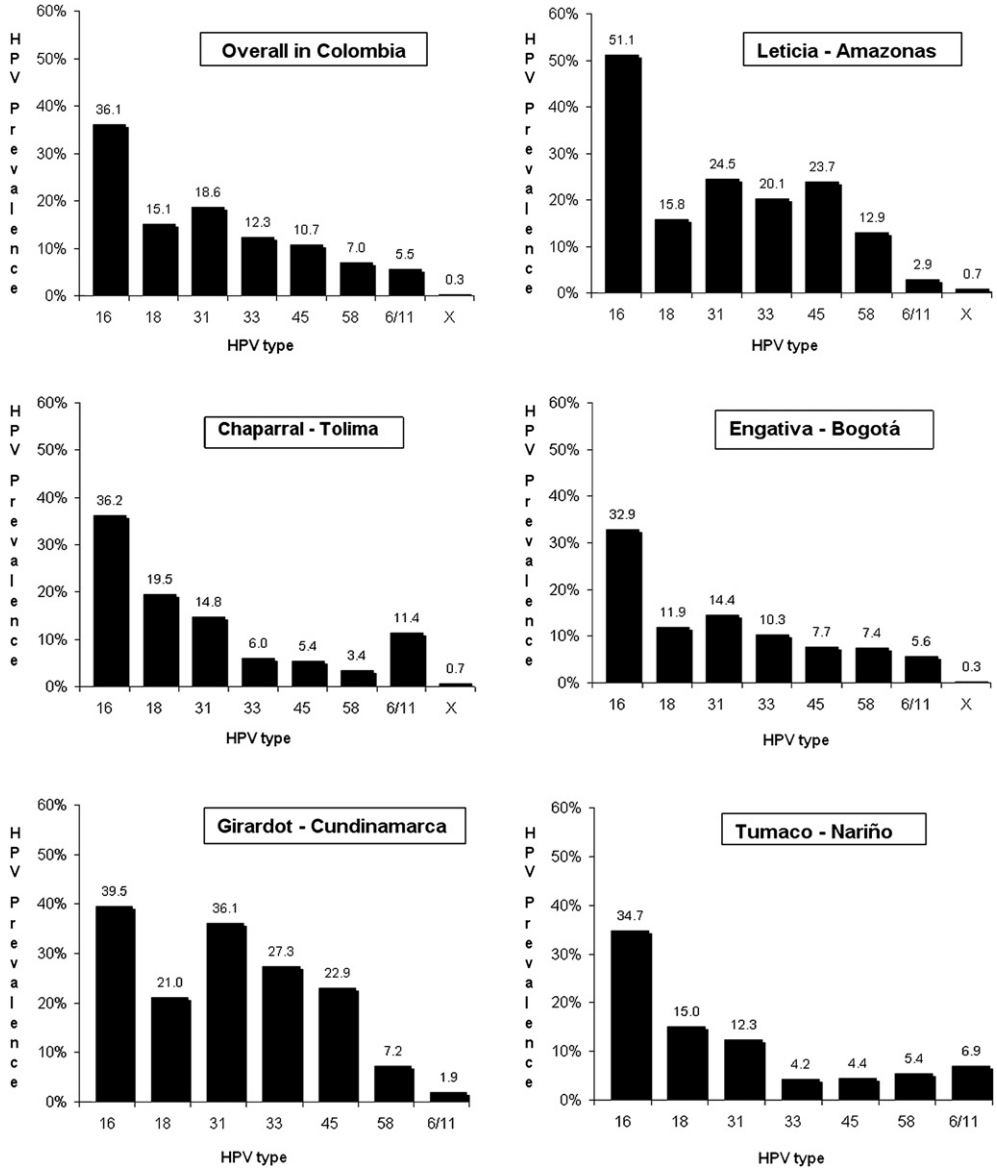

FIGURE 1. Prevalence of HPV types among 1810 Colombian women from different geographical regions.

and different factors associated with the risk of developing CC (age, contraceptive method, pregnancies, age at first intercourse, lifetime number of sexual partners, marital status, ethnicity and geographic region), the regression analysis showed that the number of pregnancies (absence of pregnancies conferred risk when compared to having had 4 or more full-term pregnancies), geographical region (the greater risk was observed in women coming from Chaparral and Leticia, compared with women coming from Engativá), and lifetime sexual partners (having had two or three sexual partners represents a lower risk compared with having had a single sexual partner) were strongly associated with any HPV infection (Table 3); these risk factors were analyzed regarding coinfection, being the results similar to those found for any infection; differences were only observed for the ethnical background (indigenous people were at greater risk compared with white people) and for geographical region, where women from Girardot or Leticia were at greater risk (Table 4).

Assessed variables remained significant when we ran the stepwise model, which included interaction terms to determine whether these had an effect in the model, as follows: pregnancies-nulliparity adjusted OR 1.56 (95% CI, 1.07–2.29), lifetime number of sexual partners (2–3) adjusted OR 0.81 (95% CI, 0.67–0.98), ethnicity-indigenous adjusted OR 2.26 (95% CI, 1.13–4.52), as well as for the

TABLE 3. Behavioral and sociodemographic factors associated with any HPV infection (single and multiple infection)

Factors	Any HPV infection n (%)	Crude ORs (95% CI)	Adjusted ORs (95% CI)
Age			
<24	115 (52.0)	1.00 (—)	
25–34	235 (50.9)	0.95 (0.69–1.32)	0.98 (0.68–1.42)
35–44	238 (47.2)	0.82 (0.60–1.13)	0.92 (0.61–1.37)
45–54	194 (48.3)	0.86 (0.62–1.43)	0.95 (0.61–1.47)
>55	95 (51.6)	0.98 (0.66–1.45)	1.04 (0.61–1.76)
Contraceptive method			
None	357 (49.1)	1.00 (—)	
Oral contraceptives	48 (51.6)	1.10 (0.71–1.69)	1.09 (0.68–1.74)
Surgery	255 (47.5)	0.93 (0.74–1.17)	0.94 (0.73–1.23)
Condom	40 (50.0)	1.03 (0.65–1.64)	1.01 (0.61–1.67)
Intrauterine device	102 (51.5)	1.09 (0.80–1.50)	1.06 (0.75–1.50)
Injectable contraceptives	51 (55.4)	1.28 (0.83–1.99)	1.20 (0.74–1.92)
Pregnancies			
None	68 (58.7)	1.47 (1.00–2.17)*	1.78 (1.04–3.04)*
1–2	305 (49.0)	0.97 (0.80–1.18)	1.14 (0.82–1.60)
3–4	309 (50.1)	1.03 (0.84–1.25)	1.18 (0.88–1.58)
>4	181 (46.8)	1.00 (—)	
Age at first intercourse			
<15	207 (47.6)	1.00 (—)	
16–17	236 (51.9)	1.20 (0.92–11.56)	1.22 (0.92–1.62)
18–19	209 (48.9)	1.06 (0.81–1.39)	1.13 (0.84–1.52)
>19	212 (48.9)	1.06 (0.81–1.39)	1.04 (0.75–1.44)
Lifetime sexual partners			
1	385 (51.7)	1.00 (—)	
2–3	376 (46.1)	0.80 (0.65–0.97)*	0.79 (0.63–0.99)*
>3	92 (51.1)	0.97 (0.70–1.35)	0.96 (0.66–1.39)
Marital status			
Single	144 (48.3)	1.00 (—)	
Married	206 (50.5)	1.09 (0.80–1.47)	1.06 (0.74–1.54)
Civil union	444 (49.5)	1.04 (0.80–1.36)	1.05 (0.76–1.46)
Separate	61 (44.2)	0.84 (0.56–1.27)	0.93 (0.59–1.47)
Widow	27 (61.3)	1.69 (0.88–3.25)	1.72 (0.82–3.59)
Ethnicity			
White	152 (50.3)	1.00 (—)	
Indigenous	30 (65.2)	1.85 (0.96–3.54)	2.03 (0.90–4.58)
Mestizo	517 (49.0)	0.95 (0.73–1.22)	1.01 (0.81–1.50)
Black	182 (48.1)	0.91 (0.67–1.24)	0.92 (0.58–1.46)
Geographic region			
Engativa	367 (46.1)	1.00 (—)	
Chaparral	81 (54.3)	1.23 (0.88–1.72)	1.66 (1.01–2.71)*
Girardot	165 (51.6)	1.01 (0.86–1.40)	1.25 (0.91–1.72)
Leticia	85 (61.1)	1.66 (1.16–2.37)*	1.82 (1.14–2.91)*
Tumaco	199 (49.0)	0.97 (0.77–1.21)	1.26 (0.83–1.93)

CI = confidence interval; HPV = human papillomavirus; OR = odds ratio.
OR adjusted for age, contraceptives method, pregnancies, age at first intercourse, number of lifetime sexual partners, marital status, ethnicity, and geographic region.
*$p < .05$.

two geographical regions: Girardot adjusted OR 1.60 (9% CI, 1.23–2.07) and Leticia adjusted OR 1.92 (95% CI, 1.28–2.88).

DISCUSSION

In this study, the frequencies of HR-HPV infection and coinfection were greater than the ones reported for other Colombian populations (2, 18). It is possible that the frequency of HPV infection was underestimated in such studies because of the use of a single primer set for detecting HPV–DNA and because the population was not as culturally diverse as the one in this study (19). Similar frequencies to the ones found here have been reported for Latin America (20–22), Africa (20), and Europe (23, 24). Moreover, other methodological aspects such as the PCR's greater sensibility for detecting viral DNA, compared with other available methods (10), and the simultaneous detection of low viral

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TABLE 4. Behavioral and sociodemographic factors associated with multiple infection

Factors	Multiple infection *n* (%)	Crude ORs (95% CI)	Adjusted ORs (95% CI)
Age			
<24	81 (70.4)	1.00 (—)	
25–34	140 (59.6)	0.90 (0.66–1.22)	0.95 (0.67–1.34)
35–44	142 (59.6)	0.77 (0.57–1.04)	0.86 (0.58–1.25)
45–54	138 (71.1)	0.90 (0.65–1.23)	0.96 (0.63–1.46)
>55	69 (72.6)	1.02 (0.70–1.48)	1.03 (0.62–1.70)
Contraceptive method			
None	226 (63.31)	1.00 (—)	
Oral contraceptives	34 (70.8)	1.17 (0.77–1.77)	1.09 (0.70–1.71)
Surgery	159 (62.3)	0.92 (0.74–1.13)	0.98 (0.76–1.25)
Condom	31 (77.5)	1.06 (0.68–1.65)	1.04 (0.65–1.66)
Intrauterine device	68 (66.6)	1.07 (0.80–1.44)	1.09 (0.78–1.52)
Injectable contraceptives	31 (60.7)	1.15 (0.77–1.71)	1.13 (0.73–1.75)
Pregnancies			
None	43 (63.27)	1.64 (1.11–2.43)*	1.67 (1.01–2.76)*
1–2	199 (65.2)	1.09 (0.85–1.39)	1.12 (0.81–1.54)
3–4	195 (63.1)	1.08 (0.85–1.38)	1.10 (0.83–1.45)
>4	120 (66.3)	1.00 (—)	
Age at first intercourse			
<15	138 (66.6)	1.00 (—)	
16–17	163 (69.0)	1.20 (0.93–1.54)	1.18 (0.90–1.55)
18–19	130 (62.2)	1.07 (0.83–1.38)	1.12 (0.84–1.48)
>19	131 (61.7)	1.07 (0.83–1.38)	1.01 (0.74–1.38)
Lifetime sexual partners			
1	268 (69.6)	1.00 (—)	
2–3	230 (61.1)	0.80 (0.66–0.97)*	0.79 (0.64–0.98)*
>3	56 (60.8)	0.93 (0.69–1.27)	0.87 (0.61–1.23)
Marital status			
Single	101 (70.1)	1.00 (—)	
Married	139 (67.4)	1.00 (0.75–1.33)	0.95 (0.67–1.35)
Civil union	271 (61.0)	0.88 (0.69–1.14)	0.93 (0.67–1.27)
Separate	37 (60.6)	0.77 (0.52–1.15)	0.81 (0.52–1.27)
Widow	18 (66.6)	1.48 (0.82–2.67)	1.51 (0.76–3.00)
Ethnicity			
White	115 (75.6)	1.00 (—)	
Indigenous	25 (83.3)	1.96 (1.07–3.59)*	2.51 (1.20–5.22)*
Mestizo	328 (63.40)	0.82 (0.64–1.05)	1.14 (0.85–1.53)
Black	100 (54.9)	0.71 (0.53–0.95)*	0.99 (0.64–1.53)
Geographic region			
Engativa	214 (58.3)	1.00 (—)	
Chaparral	50 (61.7)	1.27 (0.92–1.76)	1.45 (0.92–2.26)
Girardot	146 (88.4)	1.66 (1.29–2.15)*	1.68 (1.23–2.29)*
Leticia	66 (77.6)	2.08 (1.47–2.93)*	2.05 (1.33–3.16)*
Tumaco	105 (52.7)	0.98 (0.79–1.23)	1.10 (0.74–1.63)

CI = confidence interval; OR = odds ratio.
OR adjusted for age, contraceptives method, pregnancies, age at first intercourse, number of lifetime sexual partners, marital status, ethnicity and geographic region.
Percentages refer to the group of infected women.
*p <0.05.

loads, as well as several viral types thanks to the use of two generic primer sets (9–11), might have contributed to detect a greater viral prevalence. In addition, there are reports in literature stating that the selection of a type-specific primer can influence the results, due to preferential amplification of certain types.

HPV-16 appears to be the most prevalent type worldwide, as it is detected in approximately 50% of the cases, followed in second place by HPV-18. However, it should be noted that other authors have found a significant variation in the prevalence of other HR-HPV types among different geographical regions (25), possibly because of the influence of the biological aspects of each particular HPV type, the host immune response, as well as of each population genetics and specific environmental features (26). As expected, our study found that the most prevalent type was HPV-16, but interestingly the second most prevalent type was HPV-31 instead of HPV-18. HPV-31 has been

reported as the second most prevalent type among European females having cytological abnormalities (27, 28), which is similar to the results of this study.

The high prevalence of HPV infection found in this study does not necessarily imply a large percentage of cytological abnormalities in infected women, given that viral detection is determined by the amplification of the gene encoding the L1 protein, which is found in large amounts during active replication of HPV, and less frequently in cases of severe dysplasia due to integration of viral DNA to host cells (29, 30).

However, this study found a greater frequency of both infection and coinfection in the cities of Girardot and Leticia. The economies of these two cities rely greatly on tourism, and therefore people are more exposed to high risk sexual behaviors favoring the acquisition of multiple HPV infections and other sexually transmitted diseases (31, 32). In addition, most of the indigenous population included in this study came from the city of Leticia and showed a greater risk of presenting coinfection.

Interestingly, a HR-HPV infection rate greater than expected was here found for older women. A multivariate analysis (multiple correspondence analysis) showed that both HR-HPV infection and coinfection in this particular group were associated with an early initiation of sexual intercourse and a prolonged use of contraceptive methods (data not shown). As it has been thoroughly shown, the risk of acquiring a subsequent HPV infection increases in females already infected with HPV, being the subsequently acquired HPV types not necessarily phylogenetically related (4, 33).

Regarding socioeconomic features, indigenous ethnicity showed a high association with multiple infection, possibly attributable to cultural particularities of this ethnicity such as an early initiation of sexual activity, a reduced access to sexually transmitted disease prevention programs, or to intrinsic biological characteristics yet to be defined (34, 35). In addition, behavioral differences associated to the different ethnicities and genetic differences such as HLA allele distribution (36) cannot be ruled out as a possible explanation to the predisposition shown by the indigenous ethnicity to infection with HPV, and further studies that address this issue are required to understand better such association. Another risk factor that was found to be associated was the lifetime number of sexual partners (n = 2−3). This association has been observed in studies with Hispanic, Asian and African-American women, since it has been reported that the number of sexual partners and HPV infection vary significantly depending on the race/ethnicity (37); in addition, it has not been clearly established whether the exposure to a bigger number of viruses as the result of sexual behavior creates a competition among them in a positive or a negative manner (38).

An additional risk factor was identified when we analyzed data infection in regard to the number of pregnancies. The records showed that women with no history of previous pregnancies had a greater risk of HPV infection (single and multiple) than the ones who have had more than four full-term pregnancies. These results can be addressed from several perspectives. Because women without previous gestations are more likely to engage in sexually risky practices, such as having a large number of sexual partners. Moreover, the use of hormonal contraception methods is most frequent among women who had not given birth, which has been reported to modulate the risk of infection and disease progression, given that the virus harbors a hormone recognition fragment and therefore its behavior and distribution could be influenced by hormone changes in the female host (39).

For Latin-American populations, CC risk factors have been described as being short-termed (as age at first sexual intercourse, lifetime number of sexual partners) and long-termed (as use of oral contraceptives, high parity and cigarette smoking) (22); however, the identification of risk factor profiles in the acquisition of single or multiple infections is limited. Interestingly, our results for both types of infection did not show great differences with regard to related risk factors; this has also been observed in previous cohort studies conducted in Brazil (40) and might be attributable to the fact that all types of HPV have the same transmission vector (sexual activity) and that it has been established that coinfections depend mostly on the pre-existence of other viral types (38); according to the aforementioned, these risk factors might be contributing to a greater extent to viral persistence leading to an increased risk of developing CC than to the presence of single or multiple infections.

Additional studies are needed to provide further support to the conclusions drawn from this study, given that its cross-sectional design does not allow to fully characterize the chronology of events, such as the time elapsing between exposure to one or more than one HR-HPV and progression to significant lesions, or identifying a particular predisposition to infection by a certain HPV type when there is already another infecting HPV type.

According to our study, the frequency of all viral types varied not only among the five geographical regions, but also among the different ethnic groups herein analyzed. The results suggest that HPV infection and coinfection frequencies are strongly influenced by demographic aspects, and therefore indicate that it would be relevant to determine whether the susceptibility of a particular ethnicity is associated with intrinsic genetic features of each population, with cultural aspects and/or with particular environmental conditions that can ultimately modulate the host immune response. It would be thus important to further characterize

the risk of acquiring HPV infections in susceptible populations, in order to broaden and improve CC-prevention programs in Colombia.

In addition, because this study included a more culturally and geographically diverse population of women than other studies carried out in Colombia, the greater prevalence of HPV-31 found in this study is significant, considering that the clinical importance of this viral type has been underestimated. This viral type is considered to share a significant burden on the incidence of CC (28, 41, 42); moreover, the prevalence differences of HR-HPVs should be considered in vaccine development studies targeting Colombian populations. This is particularly relevant for anticipating the impact that immunization programs will have on these female populations, given that the currently available vaccines do not protect against all HR-HPV types (43) and that cross-immunity against other high-risk types (not included in the commercial preparations) decreases to moderate after a year of vaccination.

We thank gynecologists Adriano Mejía, Ricardo Rodríguez, Jairo Amaya, Oscar Benitez, and Luis Guillermo Cárdenas for all their collaboration in collecting samples. Our special gratitude goes to Karen Orjuela for her inspiration and dedication to this project. We also thank Catherin Marin, Carolina López, Liliana Patiño, Mónica Prias, and Marina Muñoz for their technical support; Nora Martínez for her assistance in the translation of this manuscript; and Camilo Jaimes for all his comments about the study design.

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CAPÍTULO 2

“Distribution patterns of infection with multiple types of human papillomaviruses and their association with risk factors”

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Distribution Patterns of Infection with Multiple Types of Human Papillomaviruses and Their Association with Risk Factors

Sara Soto-De Leon[1,2], Milena Camargo[1,2], Ricardo Sanchez[1,3], Marina Munoz[1,2], Antonio Perez-Prados[4], Antonio Purroy[4], Manuel Elkin Patarroyo[1,3], Manuel Alfonso Patarroyo[1,2]*

1 Molecular Biology Department, Fundacion Instituto de Inmunologia de Colombia, Bogota, Cundinamarca, Colombia, **2** School of Medicine and Health Sciences, Universidad del Rosario, Bogota, Cundinamarca, Colombia, **3** School of Medicine, Universidad Nacional de Colombia, Bogota, Cundinamarca, Colombia, **4** Mathematics Department, Universidad Publica de Navarra, Pamplona, Navarra, Spain

Abstract

Background: Infection with multiple types of human papillomavirus (HPV) is one of the main risk factors associated with the development of cervical lesions. In this study, cervical samples collected from 1,810 women with diverse sociocultural backgrounds, who attended to their cervical screening program in different geographical regions of Colombia, were examined for the presence of cervical lesions and HPV by Papanicolau testing and DNA PCR detection, respectively.

Principal Findings: The negative binomial distribution model used in this study showed differences between the observed and expected values within some risk factor categories analyzed. Particularly in the case of single infection and coinfection with more than 4 HPV types, observed frequencies were smaller than expected, while the number of women infected with 2 to 4 viral types were higher than expected. Data analysis according to a negative binomial regression showed an increase in the risk of acquiring more HPV types in women who were of indigenous ethnicity (+37.8%), while this risk decreased in women who had given birth more than 4 times (−31.1%), or were of mestizo (−24.6%) or black (−40.9%) ethnicity.

Conclusions: According to a theoretical probability distribution, the observed number of women having either a single infection or more than 4 viral types was smaller than expected, while for those infected with 2–4 HPV types it was larger than expected. Taking into account that this study showed a higher HPV coinfection rate in the indigenous ethnicity, the role of underlying factors should be assessed in detail in future studies.

Citation: Soto-De Leon S, Camargo M, Sanchez R, Munoz M, Perez-Prados A, et al. (2011) Distribution Patterns of Infection with Multiple Types of Human Papillomaviruses and Their Association with Risk Factors. PLoS ONE 6(2): e14705. doi:10.1371/journal.pone.0014705

Editor: Karin E. Peterson, Rocky Mountain Laboratories, NIAID, NIH, United States of America

Received July 19, 2010; **Accepted** January 28, 2011; **Published** February 17, 2011

Funding: This project was funded by Asociacion Investigacion Solidaria SADAR, Caja Navarra (Navarra, Spain) and the Spanish Agency for International Development Cooperation (AECID) (Project 08-CAP2-0609). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Despite the fact that "Caja Navarra" is a commercial funder, there are no conflicts regarding PLoS ONE policies on sharing data and materials. No new patents or product developments apply to this study.

* E-mail: mapatarr.fidic@gmail.com

Introduction

Cervical cancer (CC) is one of the leading causes of cancer-related death among women in developing countries [1]. Women in South and East Africa, Central America and South America are at greater risk of developing cervical cancer, with an average annual incidence of 40 cases per 100,000 women [2]. In Colombia, this disease has become a serious public health problem, with an annual incidence of nearly 18.2 cases of cervical cancer for every 100,000 women [3].

Persistent infection with one of the fifteen oncogenic types of Human Papillomaviruses (HPVs) (commonly denoted as high-risk HPV types (HR-HPV)) is considered the main risk factor associated with this disease [4,5]. Different epidemiological studies have suggested that about 50–75% of sexually active women are infected with HPV at some point of their lives [6,7]. However, distribution and prevalence of HPV infection rates vary largely between geographical regions worldwide, probably as a consequence of the numerous factors associated with HPV infection [8,9]. Even though more than 180 types of HPV have been described, 15 of them are of higher clinical interest due to their high association with malignant disease and their strong mucosal tropism. Furthermore, the burden of disease is mostly shared by 6 types (HPV-16, -18, -31, -33, -45, -58) which are commonly reported in scientific literature as responsible for the majority of lesions [9].

An important risk factor for developing cervical cancer is coinfection, defined as infection with more than one HPV type. It has been established that there is an important association between the number of viral types at the site of infection and the severity of the cervical intraepithelial neoplasia [10,11]. Of the total number of women infected with HPV, about 20–50% is believed to be infected with more than one viral type [12–14].

Other factors which contribute to the risk of developing cervical cancer have been described, including age [15]; the lifetime number of sexual partners, which has been especially associated with coinfection [10]; cigarette smoking, although data regarding

this aspect is controversial [16]; prolonged use of hormonal contraceptives (reviewed in [2,16]); and a large number of full-term pregnancies [17], among others. Recently, Almonte *et al.* divided risk factors for Latin American populations into two groups. The group of short-term risk factors included those related to sexual behaviors, such as age at first sexual intercourse, lifetime number of sexual partners and the sexual behavior of such partners; while the group of long-term risk factors comprised the use of oral contraceptives, high parity and cigarette smoking as cofactors in cervical cancer etiology [18].

Nonetheless, these data are not definitive since discrepancies concerning the factors that favor an increase in the severity of cervical intraepithelial lesions exist, which are most likely related to differences between the methodologies used for collecting samples and laboratory detection methods [19], as well as between women sociodemographic characteristics and sexual behaviors [20]. The two later discrepancies stress the importance of establishing associations between clinical and sociodemographic data with molecular findings in HPV infection events, specific for genetically diverse populations as the ones found in our country.

The present study involved 5 regions with different socio-cultural and geographical characteristics, whose CC mortality rates ranged between 3.12 and 5.67/100,000 inhabitants. These regions are: Bogota, the country's capital, with an urban population, which have access to adequate health facilities. Leticia, localized in the department of Amazonas, which is a tropical region inside the colombian jungle characterized by its proximity to Peru and Brazil, favored by high migration rates and an important ethnical diversity with a predominant indigenous background. Chaparral, located in the department of Tolima, is mainly constituted by mestizos with a sedentary life style. Another region participating in this study was Girardot, located in the department of Cundinamarca which due to its favorable weather and great proximity to Bogota has become a preferred touristic destination. It is worth noting that most of the afore-mentioned regions display a predominantly mestizo population, whereas the fifth region considered in this study, Tumaco, which is a Pacific coastal region, represents most of the black population enrolled for this work (Figure 1).

The aim of this study was to assess the number of simultaneous infections in a sample of 1,810 colombian women, which as mentioned in the previous paragraph, come from different regions. With these data, we determined epidemiological profiles of some risk factors associated with possible coinfection patterns.

Results

Of the total number of women who voluntarily agreed to participate in this study ($n = 2{,}110$), cervical samples collected from 1,815 women had optimal DNA quality according to β globin gene amplification. Five samples were excluded from the analysis taking into account that they were reported as undetermined in the specific viral type identification; therefore, 1,810 were considered for the statistical analysis. The mean age of these women was 39±12 years, being the youngest 14 years old and the oldest 77 years old. Sociodemographic and clinical data of women participating in this study are shown in Table 1.

The HPV prevalence among these women was 49.9% (CI 95%: 47.1%–51.7%), with HPV-16 being the most frequently detected

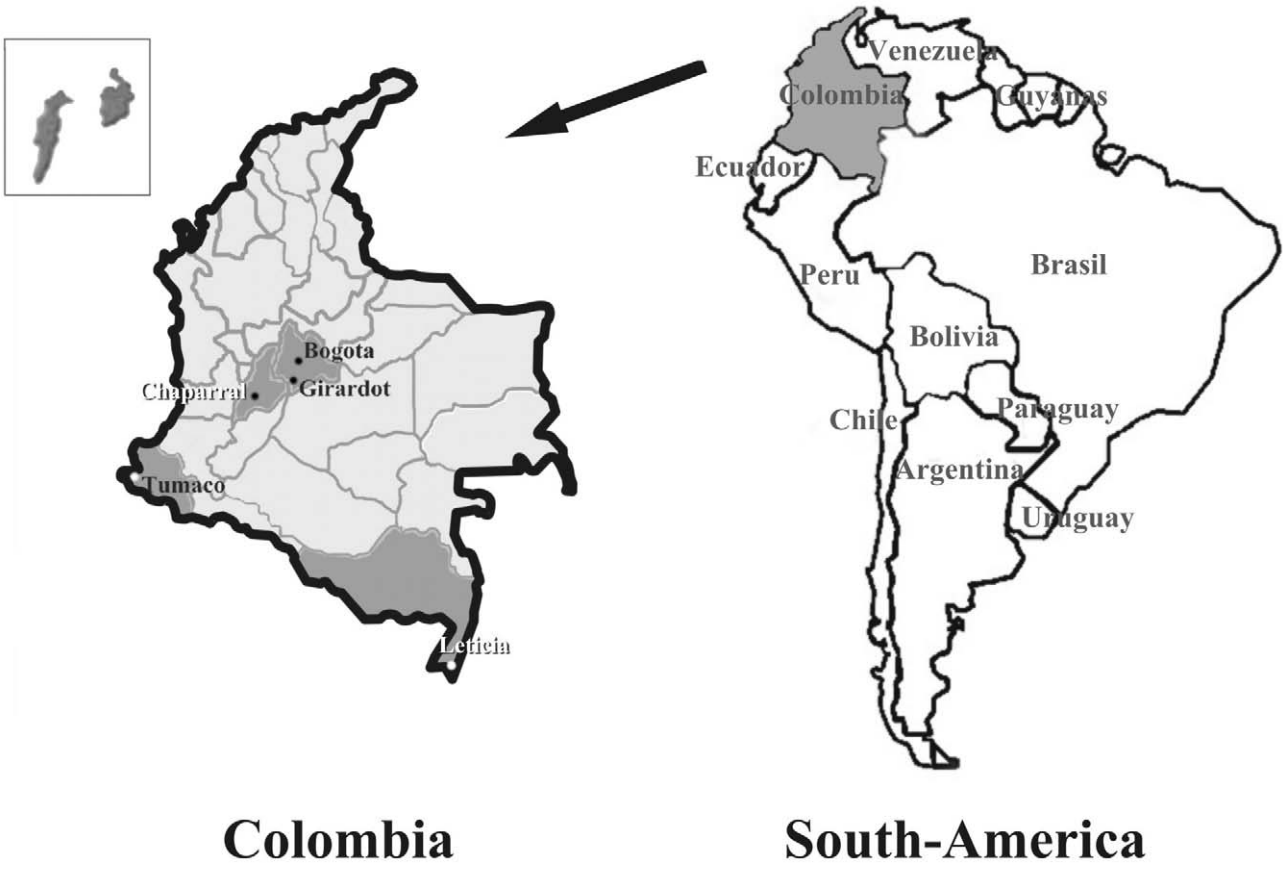

Figure 1. Geographic localization of the five populations included in this study (modified from [49]).
doi:10.1371/journal.pone.0014705.g001

Table 1. Sociodemographic characteristics, HPV DNA testing and Pap testing results for the 1810 women included in this study.

Characteristics		n (%)*
	<20	57 (3%)
Age	20–40	865 (49%)
	>40	849 (48%)
Smoking	Yes	198 (11%)
	No	1607 (89%)
Lifetime	1	745 (43%)
number of	2–3	816 (47%)
sexual partners	>3	179 (10%)
Age at first	≥18	860 (49%)
intercourse	<18	895 (51%)
	0	116 (7%)
Pregnancies	1–2	619 (36%)
	3–4	618 (35%)
	>4	388 (22%)
	White	302 (17%)
Ethnicity	Indigenous	46 (3%)
	Mestizo	1054 (59%)
	Black	378 (21%)
	None	728 (42%)
Contraceptive	Hormonal	185 (11%)
method	Surgery	536 (31%)
	Barrier	277 (16%)
	Single	299 (17%)
	Married	408 (23%)
Marital status	Civil Union	896 (50%)
	Divorced	137 (8%)
	Widow	45 (2%)
	Normal Cytology	1564 (91%)
Pap smear	ASCUS**	92 (5%)
results	LSIL†	49 (3%)
	HSIL‡	13 (1%)
	HPV-16	834 (42%)
Typing results	HPV-18	268 (14%)
for HPV-DNA	HPV-31	290 (15%)
positive	HPV-33	194 (10%)
samples§	HPV-45	172 (9%)
	HPV-58	94 (5%)
	HPV-6/11	100 (5%)
TOTAL		1810

*Categories have a size lower than 1810, given that there are missing data on the surveys.
**Atypical squamous cells of undetermined significance (ASCUS).
†Low squamous intraepithelial lesion (LSIL).
‡High squamous intraepithelial lesion (HSIL).
§Percentages correspond to infection events with each HPV type, calculated over a total of 1952 infections, given that some women were coinfected.
doi:10.1371/journal.pone.0014705.t001

type (Table 1). Of the total number of infected women, 65.2% (CI 95%: 62%–68.3%) were infected with multiple HPV types. A previous study on this population had found that infections with multiple HR-HPV types are more frequently associated with types belonging to the A7 species [14]. Compared to expected frequencies and assuming a Negative Binomial distribution, the observed number of women having a single infection (one HPV type) was significantly smaller than expected. For infections with two, three or four HPV types, observed frequencies were generally larger, while detection of more than four viral types in a single woman was much lower than expected (Table 2).

Since the likelihood–ratio test for alpha = 0 ($\chi^2(1) = 221.60$; $P = 0.000$) indicated over dispersion, a robust negative binomial regression model was applied to predict the number of infections per woman (a count outcome). This model was evaluated considering all explanatory variables (age, smoking, lifetime number of sexual partners, age at first intercourse, number of pregnancies, ethnicity, contraceptive method, marital status and cytology). The regression model that best predicted the number of HPV infecting types included ethnicity and number of pregnancies (Table 3).

The coefficients for this model are shown in Table 3, as the percentage change in the number of HPV infecting types. When all other variables were maintained constant, having had 1 or more pregnancies decreased the expected number of HPV infecting types, compared to women with no pregnancies. Regarding ethnicity, the expected number of HPV infecting types increased in indigenous women by 37.8% when compared to white women, whereas this number decreased in black and mestizo women (40.9% and 24.6%, respectively).

Discussion

The extended access to HPV vaccines against certain specific HPV types has motivated studies to determine cross-protection conferred by such vaccines and the role of multiple HPV infections in the development of cervical lesions. It has been demonstrated that there is a direct association between the severity of a lesion and coinfection events, which has been shown to depend on the number of HPV infecting types. Such association has been observed both in coinfections with HR-HPV types, as well as with low risk (LR) types [19]. Follow-up studies have reported that those women infected with one HPV type at the beginning of the study have an increased risk of acquiring a second HPV type [21].

Even though it is wide known that HPV infection prevalence varies in different regions given the population characteristics and the techniques used for detection, this study shows similar data to those reported for Latin America in the last two years, in which infection rates equal or greater than 50.0% were found [22,23]. As has been previously described, using more than one generic primer set allowed us to detect a greater number of infected women [24], as well as a better coinfection estimate [25].

The clinical importance of coinfection in cervical cancer etiology has been cause of extensive debate. There are reports of patients with multiple cervical intraepithelial lesions of different grades [20], but their association with coinfection events is still not clear. Despite coinfection is seldom reported in cervical cancer [26–28], several studies have shown an association between HPV coinfection and the increase in the lesion severity [29,30]. Although the findings mentioned above might seem contradictory, a possible explanation might be that HPV coinfection could favor the settlement of one of the infecting viruses, given that tumors are usually clonal and thus reflect only 1 HPV type. This notion is supported by previous studies where infections caused by some viral types are favored by a previous infection with other types [31]. These data, plus the partial protection of the currently

Table 2. Number of Observed and Expected data according to a Negative Binomial distribution.

Negative Binomial distribution frequencies		Number of viral types detected by PCR in the cervical sample													
		0		1		2		3		4		>4		$\chi 2$	
CHARACTERISTICS		Obs	Esp	Obs	Esp	Obs	Esp	Obs	Esp	Obs	Esp	Obs	Esp	(5df)	p =
	<20	27	26	8	14	9	8	10	4	3	2	0	1	10.74	p = 0.05
Age	20–40	434	430	161	216	150	109	73	55	40	27	7	14	12.94	**p = 0.02**
	>40	436	405	133	212	132	111	77	58	53	30	18	16	11.17	p = 0.05
Smoking	Yes	105	100	33	49	33	25	15	12	9	6	3	3	12.94	**p = 0.02**
	No	808	779	278	401	264	207	147	107	88	55	22	28	12.5	**p = 0.03**
N° Lifetime	1	362	346	115	185	128	99	83	53	48	28	9	15	11.09	p = 0.05
sexual	2–3	439	417	145	204	122	100	62	49	34	24	14	12	21.32	**p = 0.01**
partners	>3	88	87	35	45	28	23	15	12	12	6	1	3	12.5	**p = 0.03**
Age at first	≥18 years	441	431	158	215	139	107	76	54	36	27	10	13	12.94	**p = 0.02**
intercourse	<18 years	452	424	140	223	145	117	84	62	60	32	14	17	11.12	p = 0.05
	0	48	51	25	29	22	16	9	9	9	5	3	3	10.78	p = 0.06
Pregnancies	1–2	316	306	104	155	104	78	63	40	26	20	6	10	12.55	**p = 0.03**
	3–4	309	298	113	154	94	80	55	41	38	21	9	11	11.17	p = 0.05
	>4	208	191	59	97	59	49	33	25	22	13	7	6	12.55	**p = 0.03**
	White	150	131	36	74	41	42	36	24	28	13	11	8	7.2	p = 0.20
	Indigenous	17	17	4	11	8	7	9	4	6	3	2	2	4.75	p = 0.44
Ethnicity	Mestizo	539	524	186	263	177	132	92	67	51	33	9	17	12.94	**p = 0.02**
	Black	196	202	82	94	67	44	21	20	9	10	3	4	21.92	**p = 0.01**
	None	372	357	128	182	119	93	58	47	40	24	11	12	12.55	**p = 0.03**
Contraceptive	Hormonal	86	87	34	46	39	24	11	13	13	7	2	4	11.12	p = 0.05
method	Surgery	282	264	95	134	68	68	52	35	28	18	11	9	12.55	**p = 0.02**
	Barrier	136	132	42	69	50	36	34	19	14	10	1	5	11.17	p = 0.05
	Single	154	141	43	75	42	39	37	21	18	11	5	6	11.15	**p = 0.01**
Marital	Married	203	196	65	102	77	53	32	28	28	14	3	7	11.17	p = 0.05
status	Civil Union	453	447	172	224	151	112	66	56	40	28	14	14	12.94	**p = 0.02**
	Divorced	77	71	23	34	15	17	15	8	4	4	3	2	21.32	**p = 0.01**
	Widow	19	20	8	11	10	6	5	3	3	2	0	1	10.74	p = 0.05
	Normal Citology	787	758	265	391	268	201	136	104	86	54	22	28	11.17	p = 0.05
Pap smears	ASCUS**	39	44	23	23	16	12	8	7	6	3	0	2	10.88	p = 0.05
results	LSIL†	22	22	9	12	5	7	9	4	3	2	1	1	8.03	p = 0.16
	HSIL‡	5	5	1	3	1	2	4	2	1	1	1	1	4.28	p = 0.51
TOTAL		**916**	**881**	**311**	**452**	**298**	**232**	**162**	**119**	**98**	**61**	**25**	**31**	**12.55**	**p = 0.03**

*Data with $p<0.05$ are shown in bold types.
**Atypical squamous cells of undetermined significance (ASCUS).
†Low squamous intraepithelial lesion (LSIL).
‡High squamous intraepithelial lesion (HSIL).
doi:10.1371/journal.pone.0014705.t002

available vaccines, support studying multiple HPV infections more in depth, as well as determine the risk factors associated to them.

A similar HPV prevalence for each Pap smear category was found. The absence of a greater viral presence in the HSIL group might be explained by GP5+/6+ and MY09/11 primer sets' amplification targets, both of which lie within the L1 encoding gene. It has been shown that viral DNA is mainly integrated to the host genome in high-degree lesions, leading to the partial loss of the L1 ORF [32,33].

In this study, observed frequencies for the number of infecting genotypes according to a binomial negative distribution, were larger than expected for the group of women coinfected with 4 viral types or less, but smaller for the group of women with a single infection; this trend could be considered as another marker for the high frequency of coinfection present in the population studied. In contrast, the group with women coinfected with more than 4 viral types showed observed frequencies lower than expected, which could be attributed to a greater exposure of this population to the most widely distributed viral types.

A previous study reported by Chaturvedi *et al.*, has shown a similar HPV distribution pattern between HIV(+) and HIV(-) women [34], leading us to suggest that such pattern is not necessarily related to the host immunological condition, but rather

Table 3. Negative binomial regression model.

X variables	Coef.	P>z	[95% CI]*		%**
1–2 pregnancies	−0.319750	0.004	−0.537142	−0.102358	−27.4
3–4 pregnancies	−0.327596	0.004	−0.553170	−0.102022	−27.9
>4 pregnancies	−0.372914	0.004	−0.629364	−0.116465	−31.1
Indigenous	0.320354	0.041	0.013561	0.627147	37.8
Mestizo	−0.282646	0.001	−0.444652	−0.120641	−24.6
Black	−0.526085	0.000	−0.730887	−0.321284	−40.9

*CI: Confidence Intervals.
**Percent change in expected count for unit increase in X.
doi:10.1371/journal.pone.0014705.t003

to the natural history of HPV infection; however, more studies including statistical distributions are needed to shed some light on this matter.

An important conclusion obtained from the multivariate analysis was the decreased risk of acquiring more viral types in women who had given birth one or more times. High parity has been usually associated with the risk of developing cervical cancer, based on the notion that a large number of births increases the number of lesions in the birth canal or causes immunosuppression in such area (reviewed in [2]). However, according to a study conducted by Molano *et al.*, the risk of acquiring infections with high risk HPV types, as well as coinfections, tends to diminish with the number of births [13]. This agrees with the results of the present study, which show that the risk of being infected with a large number of HPV types decreased to 31.1% in women who had given birth 4 or more times, compared to women who had not given birth. An explanation to this observation could be associated with riskier sexual behaviors in the latter group of women (e.g. large number of sexual partners during a woman's lifetime). However, this conclusion should be taken with caution because there are not sufficient studies relating the number of births with coinfection, or coinfection with hormonal conditions after labor.

Another important aspect to consider is the increase of a 37.8% in the number of infecting HPV types found in women of indigenous ethnicity, and the decrease of 24.6% and 40.9% in women of mestizo and black ethnicities, respectively, compared to white women. These findings could be influenced by sociocultural and demographic conditions intrinsic to these ethnic groups [35]. The indigenous ethnicity appears to play an important role in the association of infection, possibly due to cultural characteristics, such as limited access to prevention and promotion programs of cervical cancer and sexually transmitted diseases, early start of sexual relationships and childbirth, or intrinsic biological characteristics of this particular ethnia, which contribute to the immune system modulation and might suppress the immune response, thus facilitating the viral infection [36,37].

Regarding this later aspect, few studies have focused on the susceptibility to coinfection in different ethnic groups. There are reports showing that black women display a certain degree of protection against HPV infection when compared to hispanic ones [38,39], agreeing with our findings. However, other studies carried out with black women reported that infections found in this population are higher [40]. There are no studies which assess the true HPV prevalence in black women in different geographical regions, since most of recent studies carried out with black women are focused in vaccination and post-vaccination monitoring.

Taking into account a study carried out by Kenney in 1996, in which risk factors associated to infections were assessed in different ethnic groups, it can be inferred that black women have more risk to acquire infections due to their sexual behavior. In average, black women have more life time sexual partners than hispanic women, their actual sexual partners have had more lifetime sexual partners and cigarette smoking is higher in this population. On the other hand, black women that acquire infection have been taking oral contraceptives longer than hispanic women who also develop infection [36]. It is necessary to corroborate these statements in the light of statistical significance. HPV infection susceptibility in black women has been also contradictory from the host genetic background, since there are studies that reported HLA-class-II DQB1*03 alleles both as a risk factor [41,42] and a protective factor [43]. Further studies are required to establish the real role of infection in this ethnic group.

For Latin America in particular, some studies have examined the role of genes, especially HLA haplotypes in the risk of acquiring HPV infections [44–46]. Studies conducted in Brazil have reported major histocompatibility complex alleles involved in susceptibility and protection against HPV infections [47]. In Colombia, no studies have been conducted to identify autochthonous genetic factors related to the predisposition to acquire single or multiple HPV infections to date.

We found a strong association between multiple HPV infections and indigenous ethnicity in a previous study, where the same population described here was analyzed [14]; however, no association with other ethnicities was found. The different outcomes might have been due to a different dependant variable, which in this case is the number of HPV infecting types. Low-risk infections were not considered in the previous study.

This study reports an important association between the risk of acquiring infection with multiple HPV types and the indigenous ethnicity, which highlights the importance of focusing studies in this vulnerable population. Nevertheless the transversal design of this study prevents detecting neither acquisition patterns of more than one HPV type nor its relationship with the different risk factors, which make it relevant to conduct longitudinal studies to detect the moment when the acquisition of other HPV types occur and determine if infection events take place simultaneously. Here we report that the infection cases observed with 2 to 4 HPV types are greater than the expected, which suggests the importance of carrying out an analysis of infecting species. Even though we did not assess the distribution of coincident HPV types in our study, our group has reported two studies in which this topic is discussed [14,48].

The analysis carried out in this study is important since few studies have compared the risk factors associated with single and multiple HPV infections in socioculturally diverse populations as the one existing in Colombia.

Materials and Methods

Study population and ethical considerations

This study enrolled women living in five geographical regions of Colombia attending to their cervical screening consult between April and September of 2007. These five populations have been described elsewhere [14]. Prior to undergoing gynecological examination, all women signed a written informed consent and filled out a questionnaire regarding sociodemographic characteristics, sexual behaviors and risk factor data (Table 1). An informed consent was signed by a parent or guardian in the case of children under age. Each women voluntarily agreed to provide a sample of cervical epithelium which was analyzed by Papanicolau testing and HPV–DNA detection. This study was conducted with the supervision and approval of each institution's Ethics Committee,

as follows: the Ethics Committee of Fundación Instituto de Inmunología de Colombia, Bioethics Committee of the League Against Cancer - Amazon Sectional, The Ethics Committee of Nuevo Hospital San Rafael E.S.E - Girardot, Bioethics Committee of Hospital San Juan Bautista de Chaparral E.S.E, Hospitalary Ethics Committee of Hospital de Engativá Nivel II and Ethics Committee of Hospital San Andres, Tumaco E.S.E.

Detection of HPV DNA by PCR amplification

Cervical samples were collected with a cytobrush and stored in 95% ethanol at 4°C [49]. DNA from these samples was digested with lysis buffer containing 10 mM Tris–HCl (pH 7.9), 0.45% Nonidet–P-40, 0.45% Tween 20 and 60 µg/mL of Proteinase K (Invitrogen, California, USA), first at 60°C for 1 h and then at 95°C for 10 min [50]. A 2.7-µL aliquot of each processed sample was amplified by PCR using the human β-globin GH20/PC04 primers to check DNA integrity [51] and two HPV generic primer sets were employed for detection, to prevent underestimation in the determination of viral prevalence caused by the use of a single HPV identification set [52]. These sets are: GP5+/GP6+ which allows detecting low viral loads [51,53] and MY09/MY11 which has higher sensitivity for detecting infections with more than one viral type [25]. Additionally, these generic primer sets are easily implemented and increase robustness and sensibility to epidemiological studies [24,54]. Despite the advantages obtained, it is worth noting that the use of multiple primer sets can also increase contamination, which is why protocols aimed at minimizing this contamination must be followed. In the present study, negative and positive controls were run every 46 samples, and whenever either control gave an unexpected result, the whole series was repeated. Samples testing positive with one or both generic primer sets underwent PCR amplification with type-specific primers for HPV-6/-11, -16, -18, -31, -33, -45 and -58 annealing within the E5, E6 and E7 regions [4,24,55,56], Studies carried out in Latin America have found that the 6 high-risk viral types included in this study are present in the 90% of cases of cervical cancer [57]. Synthetic genes encoding HPV-18, -31, -45 and -58 early regions and HPV-6/-11, -16 and -33 infected samples were used as positive controls for type-specific PCR reactions [58]. This PCR procedure was carried out in duplicate and DNA-free water was used as negative control to rule out DNA contamination.

Statistical analysis

Descriptive statistics were used to evaluate demographic and clinical characteristics of the population. The expected number of coinfecting genotypes was estimated assuming a negative binomial distribution. Although previous studies used a Poisson model to evaluate the fit of data [34], in our sample the over dispersion coefficient alpha was significantly different from zero for all of the variables included in the analysis. The difference between observed vs. expected frequencies was evaluated using a one-way χ^2 Goodness-of-fit test.

In order to evaluate which explanatory variables (age, smoking, lifetime number of sexual partners, age at first intercourse, number of pregnancies, ethnicity, contraceptive method, marital status and cytology) were associated with the number of coinfections (dependent variable), a negative binomial model was used instead of a Poisson distribution, since this model does not consider the existence of over dispersion (in our data the mean/variance ratio was 0.7) [59]. Additionally, a likelihood ratio test was used to compare the relative fit of the Poisson model to the negative binomial model. The negative binomial model assumes that the number of coinfections is a count outcome. The use of this model is preferred over other tests that estimate ORs, given both the count outcome and the high frequencies of the expected outcomes [60]. All the statistical analyses were performed using STATA 9®.

Acknowledgments

We would like to sincerely thank chief nurses Maritza Arriero, Daira Mendez and Eva Vega and gynecologists Patricia Otálora, Alcides Bracho and Alveiro Ramos for their support to this project. We thank also Dyna Gordillo, Andrés Moreno and Laura Paipa for their technical support, and Camilo Jaimes and Nora Martínez for all their comments and suggestions on this text. Gisselle Rivera collaborated in the translation of this manuscript.

Author Contributions

Conceived and designed the experiments: SSDL MC RS MM MEP MAP. Performed the experiments: SSDL MC MM. Analyzed the data: SSDL MC RS APP AP MEP MAP. Wrote the paper: SSDL MC RS MEP MAP.

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CAPÍTULO 3

"Detection by PCR of human papillomavirus in Colombia: Comparison of GP5+/6+ and MY09/11 primer sets"

Journal of Virological Methods 178 (2011) 68–74

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Detection by PCR of human papillomavirus in Colombia: Comparison of GP5+/6+ and MY09/11 primer sets

Milena Camargo[a,b], Sara Soto-De Leon[a,b], Ricardo Sanchez[a,c], Marina Munoz[a,b], Erika Vega[a,b], Magda Beltran[a,b], Antonio Perez-Prados[d], Manuel Elkin Patarroyo[a,c], Manuel Alfonso Patarroyo[a,b,*]

[a] Molecular Biology Department, Fundación Instituto de Inmunología de Colombia, Bogotá, Colombia
[b] School of Medicine and Health Sciences, Universidad del Rosario, Bogotá, Colombia
[c] School of Medicine, Universidad Nacional de Colombia, Bogotá, Colombia
[d] Mathematics Department, Universidad Pública de Navarra, Pamplona, Spain

Article history:
Received 15 March 2011
Received in revised form 9 August 2011
Accepted 14 August 2011
Available online 22 August 2011

Keywords:
Human papillomavirus (HPV)
GP5+/6+
MY09/11
Colombia
Multiple infections

A B S T R A C T

The aims of this study were to determine the prevalence of HPV infection and evaluate the concordance and performance of two primer sets for detecting single and multiple viral infections. A total of 1810 Colombian women were enrolled in the study, and molecular, cytological and epidemiological analyses were performed. Both concordance and performance of two different PCR amplification primer sets (GP5+/6+ and MY09/11) were assessed. The results showed that 60.2% of females with positive HPV DNA were infected by more than one viral type. The OR for multiple infections was 18.2 when using the MY09/11 primer set and 6.52 with the GP5+/6+ primer set. The results also showed an association between GP5+/6+ positivity and the severity of the disease regarding the cytological findings. It was also found that using a single primer set led to underestimating the prevalence for HPV infection. The simultaneous use of these primer sets is an important tool for the detection of HPV DNA, being equally relevant for identifying multiple infections and low viral DNA copies. This study highlights the importance of suitable assessment of HPV epidemiological profiles; screening programs must also be strengthened to broaden the coverage of the most vulnerable populations.

1. Introduction

There is strong evidence implicating some human papillomaviruses (HPVs) with the induction of carcinogenesis, especially of cervical cancer which is the second most common type of cancer in females worldwide. More than 100 types of HPVs have been identified to date, but only 40 types are known to infect the genital tract (de Villiers et al., 2004).

The polymerase chain reaction (PCR) has been used extensively for HPV typing in many clinical and epidemiological studies because of its high sensitivity for detecting HPV DNA. Nevertheless, an exhaustive identification of HPV types, based solely on PCR, requires a large number of reaction per sample due the high degree of genetic heterogeneity amongst HPV types (van Doorn et al., 2006).

Different primer systems targeting relatively conserved nucleotide sequences have been developed with the aim of detecting a wide spectrum of HPV types. The sets used most frequently at present are GP5+/6+, MY09/11, PGMY and SPF10 (Iftner and Villa, 2003). The use of one or more primer sets has been recommended when designing PCR-based HPV detection methods (Karlsen et al., 1996; Remmerbach et al., 2004); this should ensure better understanding of the natural history of this particular infection and increase epidemiological studies' sensitivity. The use of more than one primer set has been shown to offer better robustness and sensitivity for detecting multiple HPV infections (i.e. infection with more than one type of HPV) as well as a low viral copy number, compared to the use of a single primer set which, in some cases, has led to underestimating the prevalence of HPV in cervical samples (Fuessel Haws et al., 2004; Iftner and Villa, 2003).

The present study was aimed at evaluating the presence of HPV DNA by using two primer sets annealing in a conserved region of the L1 gene. GP5+/6+ has been reported as a convenient method for detecting low viral loads (Remmerbach et al., 2004) whilst MY09/11 has greater sensitivity for detecting coinfections. Both primer sets

* Corresponding author at: Molecular Biology Department, Fundacion Instituto de Inmunologia de Colombia, Carrera 50 # 26-20, Bogota, Colombia.
E-mail addresses: milena_camargo@fidic.org.co (M. Camargo), sara_soto@fidic.org.co (S. Soto-De Leon), rsanchezpe@unal.com.co (R. Sanchez), marina_munoz@fidic.org.com (M. Munoz), erikavega77@gmail.com (E. Vega), maybe.leon@gmail.com (M. Beltran), aperez@unavarra.es (A. Perez-Prados), mepatarr@mail.com (M.E. Patarroyo), mapatarr.fidic@gmail.com (M.A. Patarroyo).

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doi:10.1016/j.jviromet.2011.08.014

Table 1
The primers used in this study.

Type	Primer	Primer sequence (5′–3′)	Size (bp)	Region
GH20	Pr1	GAAGAGCCAAGGACAGGTAC	268	
PC04	Pr2	CAACTTCATCCACGTTCACC		
GP5+	Pr1	TTTGTTACTGTGGTAGATACTAC	150	L1
GP6+	Pr2	GAAAAATAAACTGTAAATCATATTC		
MY09	Pr1	CGTCCMARRGGAWACTGATC	450	L1
MY11	Pr2	GCMCAGGGWCATAAYAATGG		
HPV-16	Pr1	TCAAAAGCCACTGTGTCCTGA	120	E6
	Pr2	CGTGTTCTTGATGATCTGCAA		
HPV-18	Pr1	CGACAGGAACGACTCCAACGA	202	E6–E7
	Pr2	GCTGGTAAATGTTGATGATTAACT		
HPV-31	Pr1	CTACAGTAAGCATTGTGCTAT	155	E5
	Pr2	ACGTAATGGAGAGGTTGCAATAACCC		
HPV-33	Pr1	AACGCCATGAGAGGACACAAG	212	E7
	Pr2	ACACATAAACGAACTGTGGTG		
HPV-45	Pr1	ACGGCAAGAAAGACTTCGCA	134	E6–E7
	Pr2	CACAACAGGTCAACAGGATC		
HPV-58	Pr1	CGAGGATGAAATAGGCTTGG	109	E7
	Pr2	ACACAAACGAACCGTGGTGC		
HPV-6/11	Pr1	TGCAAGAATGCACTGACCAC	334	E6–E7
	Pr2	TGCATGTTGTCCAGCAGTGT		

have been used extensively in numerous HPV identification studies (Qu et al., 1997). The study has evaluated the concordance and performance of these two primer systems for detecting HPV DNA in single and multiple infections. Similar studies have been carried out in Colombia; however, the present work has included cytology samples collected from a heterogeneous group of women from different economic, socio-demographic and cultural backgrounds whose epidemiological profiles have not been described previously and who are considered to be vulnerable populations having limited access to healthcare services.

2. Materials and methods

2.1. The population studied

This study involved 1810, 14–77 year-old females (mean age = 38.9, SD = 11.9) from five Colombian departments who attended regular cervical cancer screening sessions between April and September 2007. This population consisted of females attending the League Against Cancer, Leticia, Amazonas ($n = 140$), Hospital San Juan Bautista, Chaparral, Tolima ($n = 148$), Hospital Engativa, Bogotá ($n = 796$), Hospital San Rafael, Girardot, Cundinamarca ($n = 321$) and Hospital San Andrés, Tumaco, Nariño ($n = 405$). Stata Software (version 11) was used for estimating population size (1778 individuals, 95% confidence interval, plus or minus 0.02, estimated proportion was 0.25) (Mendez et al., 2005). The number of samples was adjusted in line with a proportional allocation in which the sample size depended on the number of cytologies being taken in each department.

2.2. Ethical approval

All the women were informed about the study by the nurses coordinating the project and then gave their written consent. They all filled out a questionnaire regarding their socio-demographic characteristics, sexual behaviour and risk factor data before undergoing a gynaecological examination. Each participating institution's Ethics Committee approved and supervised all the procedures carried out in this study.

2.3. Collecting cervical samples and PCR-based HPV detection

A cytobrush was used for collecting the cervical samples for Papanicolau testing and HPV-DNA detection; they were stored at 4 °C in 95% ethanol until processing. The Pap test cytological findings were classified according to the Bethesda system. The PCR assay samples were digested in lysis buffer containing 10 mM Tris–HCl (pH 7.9), 0.45% Nonidet P-40, 0.45% Tween 20 and 60 μg ml Proteinase K, first at 60 °C for 1 h and then at 95 °C for 10 min (Nelson et al., 2000).

The human β-globin housekeeping gene was amplified in all samples using GH20/PC04 primers to check DNA integrity (Table 1). The PCR mix contained: 1× amplification buffer, 100 μM each dNTP, 2.5 mM $MgCl_2$, 1 U Taq Polymerase (Bioline, MA, USA), 1 μM of each primer and 0.8 μg μl bovine serum albumin (BSA) as buffer additive (final volume = 10 μl) (Kreader, 1996). The thermal cycling conditions consisted of initial denaturing for 10 min at 94 °C, followed by 35 amplification cycles for 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, followed by a final extension step for 7 min at 72 °C (de Roda Husman et al., 1995).

The HPV DNA was detected using generic GP5+/6+ and MY09/11 primer sets annealing in the L1 gene (Table 1) according to published protocols (de Roda Husman et al., 1995; Manos et al., 1989) which had some modifications made to it. The GP5+/6+ PCR assays were carried out in a 20 μl volume mixture containing: 1× amplification buffer, 100 μM each dNTP, 3 mM $MgCl_2$, 1 U Taq polymerase (Bioline, MA, USA) and 2 μM of each primer. Thermal cycling conditions consisted of initial denaturing for 10 min at 94 °C, followed by

Table 2
Comparing HPV detection using two generic primers in single and multiple infections.

HPV infection status ($n = 894$)	GP5+/6+		MY09/11	
	Negative n (%)[a]	Positive n (%)[a]	Negative n (%)[a]	Positive n (%)[a]
Single infections (n = 356)	169 (47.5)	187 (52.5)	85 (23.9)	271 (76.1)
Multiple infections ($n = 538$)	269 (50.0)	269 (50.0)	110 (20.4)	428 (79.6)
Total	438	456	195	699

[a] Total percentages were calculated by rows.

40 amplification cycles for 1 min at 94 °C, 2 min at 40 °C and 1.5 min at 72 °C, followed by a final extension step for 7 min at 72 °C (de Roda Husman et al., 1995). The PCR assays with MY09/11 primers were carried out in a 20 μl volume mixture, exactly as described above. Thermal cycling conditions consisted of initial denaturing for 5 min at 94 °C, followed by 40 amplification cycles for 30 s at 94 °C, 1 min at 45 °C and 1 min at 72 °C; a final extension step lasted 7 min at 72 °C. Positive and negative controls were used in each assay to assess whether the DNA was contaminated. The PCR amplification using generic primers was carried out twice, at different times, to rule out sample contamination and reduce the number of false positives (Remmerbach et al., 2004). The amplification products obtained from all the PCRs (5 μl) were run on 2% agarose gels stained with SYBR safe (Invitrogen, CA, USA) and then visualised on an ultraviolet transilluminator.

Each set of generic primers' (GP5+/6+ or MY09/11) analytical sensitivity was only calculated for HPV 16 using plasmid DNA, meaning that these values could not be extrapolated to other HPV types. Serial dilutions of 100 ng of plasmid DNA containing the HPV-16 *L1* gene were thus used; these were extracted from transfected Sf21 cells. This procedure was carried out in duplicate and the same DNA was used as positive control.

The samples that proved positive by generic primers (when using one or both sets) were then tested with type-specific primers which had been designed to anneal in early protein genes (E5–E7) (Table 1) (Karlsen et al., 1996; Lee et al., 2001; Sotlar et al., 2004) for genotyping the two most prevalent low-risk types (HPV-6/11) and six high-risk types associated with ~90% of cervical cancers reported in Latin-America and worldwide (i.e. HPV-16, -18, -31, -33, -45, -58) (Parkin et al., 2008; WHO/ICO, 2010). The high-risk types mentioned above had the highest incidence and prevalence according to previous studies carried out in Colombia (Molano et al., 2002).

2.4. Statistical analysis

The concordance between results obtained with the two generic primer sets (GP5+/6+ and MY09/11) was assessed by using kappa statistics (κ) with 95% confidence intervals (95% CI). The difference between the percentages of cases detected by each method was analysed by applying the McNemar chi-square test. The strength of the association between each method and the variables of interest, such as coinfection and cytology findings, were measured using ORs (95% CI) and adjusting OR by logistical regression. The relationship between cytological findings and detection by each primer set was explored by using the test of trends in odds ratio (normal cytology was used as reference category). The diagnostic value for each primer set for detecting infection and coinfection was assessed by calculating their sensitivity, specificity and area under their corresponding receiver operating characteristic (ROC) curves. Areas under the ROC curves were compared by using chi square methods. Each estimator was reported with its 95% confidence interval and the hypotheses were tested at 5% significance level. STATA 9 Software was used for all statistical analysis.

3. Results

All the 1810 samples were β-globin gene positive (268-bp DNA fragment) and thus were suitable for further PCR analysis. HPV DNA was detected in 894 (49.4%) of the samples having good DNA quality (positive for β-globin gene); 699 (38.6%) of these were detected using MY09/11 and 456 (25.2%) using GP5+/GP6+ (Table 2) whilst 261 (14.4%) samples were detected with both generic primer sets. Coinfection was detected in 538 (60.2%) out of 894 HPV

Table 3
Comparison of HPV detection using the GP5+/6+ and MY09/11 primers regarding cytological findings.

Pap smear result[a] (n = 1718)	GP5+/6+		MY09/11		Crude ORs (95% CI)		Adjusted ORs (95% CI)[b]	
	Negative n (%)	Positive n (%)	Negative n (%)	Positive n (%)	GP5+/6+	MY09/11	GP5+/6+	MY09/11
Normal cytology (1564)[c]	1186 (75.8)	378 (24.2)	952 (60.9)	612 (39.1)	–	–	–	–
ASCUS (92)	61 (66.3)	31 (33.7)	52 (56.5)	40 (43.5)	**1.59 (1.01–2.49)**	1.19 (0.78–1.82)	**1.82 (1.10–2.99)**	1.46 (0.91–2.35)
LSIL (49)	26 (53.1)	23 (46.9)	26 (53.1)	23 (46.9)	**2.77 (1.56–4.93)**	1.37 (0.77–2.43)	**2.85 (1.53–5.27)**	1.24 (0.67–2.30)
HSIL (13)	6 (46.2)	7 (53.8)	8 (61.5)	5 (38.5)	**3.66 (1.21–10.9)**	0.97 (0.31–2.98)	**4.80 (1.47–15.6)**	1.34 (0.41–4.36)
Total	1279 (74.4)	439 (25.6)	1038 (60.4)	680 (39.6)				

Percentages were calculated by rows.
Values in bold had $p < 0.05$.
Abbreviations: ASCUS, atypical squamous cells of undetermined significance; LSIL, low grade squamous intraepithelial lesion; HSIL, high grade squamous intraepithelial lesion.
[a] Female samples were classified according to the Bethesda system.
[b] OR adjusted for age, number of pregnancies, age at first intercourse, number of lifetime sexual partners, ethnicity, geographical region, contraceptive method used. Confidence intervals for crude and adjusted ORs are shown in parentheses.
[c] Normal cytology was used as reference category.

Table 4
Crude and adjusted odds ratios (ORs) for HPV detection, using two generic PCR primers, in samples with known co-infections.

	Crude ORs (95% CI)		Adjusted ORs (95% CI)[a]	
	GP5+/6+	MY09/11	GP5+/6+	MY09/11
Negative for multiple infections[b] ($n = 1272$)	–	–	–	–
Positive for multiple infections ($n = 538$)	5.8 (4.53–7.42)	14.3 (10.67–19.34)	6.52 (5.03–8.47)	18.2 (13.58–24.50)

All p values were <0.05.
[a] OR adjusted for age, number of pregnancies, age at first intercourse, number of lifetime sexual partners, ethnicity, geographical region and contraceptive method used.
[b] Negative for multiple infections was used as the reference category.

DNA positive samples. The results of this study had a 5×10^{-6} ng detection limit for GP5+/GP6+ and 2×10^{-3} ng for MY09/11.

As can be observed in the Papanicolau test results (Table 3), only 92 cervical samples were unsatisfactory and they were thus excluded from the analysis. The OR values for the association between GP5+/GP6+ and the severity of the disease according to the cytological findings revealed a significant trend for detecting HPV DNA with this primer set (test of trends in odds: chi^2 (1) = 21.87, $p = 0.00$) which remained significant when ORs were adjusted. The same association with MY09/11 also showed an increasing trend; however, such trend was not statistically significant (test of trends for odds: chi^2 (1) = 1.20, $p = 0.27$).

The case detection percentages with each primer set revealed statistically significant differences (McNemar chi^2 (1) = 196.53, $p = 0.000$). The kappa statistics revealed 65% concordance between both primer systems ($\kappa = 0.22$; 0.16–0.25, 95% CI) Regarding HPV infected females, total concordance became reduced to 27% in the single infection group ($\kappa = -0.46$; −0.54 to 0.38, 95% CI) and to 30.4% in the coinfection group ($\kappa = -0.42$; −0.48 to 0.36, 95% CI). Concordance between both primer systems was 69.7% in the group of females suffering cytological abnormalities ($\kappa = 0.38$; 0.23–0.52, 95% CI) whereas it was 63.9% ($\kappa = 0.19$; 0.14–0.24, 95% CI) in the normal cytology group.

The GP5+/6+ set had 51% sensitivity for detecting HPV infections (ROC area = 0.75; 0.73–0.77 95% CI) whereas MY09/11 had 72.2% sensitivity (ROC area 0.89; 0.87–0.90 95% CI); both systems had 100% specificity. A comparison between the areas under the ROC curve for each primer system revealed significantly higher values for MY09/11 in detecting HPV infections (chi^2 (1) = 104.04, $p = 0.00$).

However, when both methods were analysed regarding their performance for detecting HPV positivity in co-infected samples, GP5+/6+ had 50% sensitivity and 85.3% specificity (ROC area = 0.67; 0.65–0.7 95% CI) whereas MY09/11 had 79.6% sensitivity and 78.7% specificity (ROC area = 0.79; 0.77–0.81 95% CI). The crude and adjusted ORs were calculated for evaluating the ability of each primer set to detect either single or multiple infections. The OR results regarding coinfection detection were 18.2 when using the MY09/11 primer set and 6.52 when using the GP5+/6+ primer set (Table 4). Comparison of the areas under the ROC curves showed that the MY09/11 set had significantly higher values for detecting coinfections (chi^2 (1) = 40.43, $p = 0.00$).

All samples that had a positive result for viral DNA presence (894 that amplified in GP, MY or both PCRs) were amplified successfully by type-specific primers, thereby revealing that HPV-16 was the most prevalent type as it was detected in 654 females (36.1%), followed, in decreasing order, by HPV-31, detected in 336 (18.6%), HPV-18 detected in 274 (15.1%), HPV-33 detected in 223 (12.3%), HPV-45 detected in 193 (10.6%), HPV-58 detected in 127 (7.0%) and HPV-6/11 detected in 100 females (5.5%).

4. Discussion

Two generic primer sets were used in the present study for detecting HPV DNA in Colombian females which led to a higher prevalence of HPV infection was found compared to previous studies carried out in Colombia. A previous study where a single primer set was used for viral identification in an urban population only showed 14% HPV prevalence (Molano et al., 2002). Another study that included both rural and urban areas in a culturally diverse, low educational level population revealed a higher prevalence of viral infection (36%) (Sierra-Torres et al., 2006) even though such prevalence was lower than that reported here.

A higher prevalence of HPV infection has been reported in several studies carried out in around the world. African countries such as Comoros, the Congo, Djibouti, Eritrea and Ethiopia have reported a prevalence of infection ranging from 23.0% to 35.4% (WHO, 2007) and 45.3% and 68.9% prevalence has been reported for France and Italy (Bello et al., 2009) whilst some Latin-American countries (Costa Rica, Honduras, Nicaragua and Panama) sharing socio-demographic characteristics similar to those described in this study, have reported 20.5–51.0% viral prevalence (Ferrera et al., 2000; Herrero et al., 2008; Safaeian et al., 2007; Tabora et al., 2009; WHO, 2007). The conclusions drawn from the present study agree with studies indicating that the use of a single primer set leads to underestimating the prevalence of HPV infection, mainly in cases of multiple infection (Fuessel Haws et al., 2004).

Women infected by HPV were detected more efficiently by MY09/11 than by GP5+/6+ according to the analytical sensitivity results calculated for each primer set. MY09/11 was the more efficient system for detecting females infected with more than one HPV type, since 428 out of 538 (79.6%) infected females were detected, whereas only 269 (50%) of the same females were detected by GP5+/6+. This finding agreed with previous reports (Qu et al., 1997) and was consistent with MY09/11 consisting of a system of degenerated primers thereby promoting the identification of multiple infections (Chaiwongkot et al., 2007).

Although it has been reported that the concordance between two generic primer sets decreases in patients having coinfection (Qu et al., 1997), this study also had low concordance in the single infection group. Concordance between both primer sets increased in females having abnormal cytological findings (this being higher for GP5+/6+), thereby agreeing with previous reports. Such relationship between lesion severity and HPV detection is consistent with the HPV life-cycle since viral DNA remains integrated within a host cell's DNA when lesion severity is high; this can result in the loss, truncation or poor transcription of the L1 gene (Depuydt et al., 2007). The GP5+/6+ set is thus more sensitive for detecting a low viral copy number of HPV DNA, even if the L1 gene is truncated, because the amplification product obtained with this primer set is just one third of that obtained when using MY09/11 (Sotlar et al., 2004). False negative results when using the MY09/11 set have been associated with the loss of the L1 ORF during HPV DNA integration into host-cell DNA (Depuydt et al., 2007).

Many commercial systems for the generic identification of HPV-infected samples are available, such as PGMY-LB (Roche Molecular Systems Inc., Branchburg, NJ), SPF10-LiPA25 (Labo Bio-Medical 221 Products, Rijswijk, the Netherlands), the Amplicor HPV test and the LINEAR ARRAY HPV genotyping test (both from Roche) and PapilloCheck HPV-Screening Test (Greiner Bio-One GmbH, Frickenhausen, Germany); these identification systems' performance

Table 5
Advantages and disadvantages of different HPV detection techniques in clinical samples.

Method of HPV detection	Country	Population	HPV prevalence %	Sensitivity %	Specificity %	Advantages	Disadvantages	Reference
GP5+/6+	Greece	1270 liquid-based cytology	31.3	100	70.2 (CIN2) 69.6 (CIN3)	Offers higher sensitivity, especially with samples containing low copy numbers. Due to their low cost and easy implementation, they have been widely used in clinical and epidemiological studies	Can be ineffective in the amplification of some HPV types such as HPV-53 and -61. Not suitable for type-specific identification	Tsiodras et al. (2010)
	Russia	1511 gynaecological outpatients	36.6	74.0	64.1			Kulmala et al. (2004)
	Colombia	1810 gynaecology outpatients	25.2	51	100			This study
MY09/11	Australia	834 cervical specimens	37.4	63.1 (HGS test) 64.7 (hcII)	90.6 (HGS test) 84.6 (hcII)	Detects more than 25 genotypes simultaneously. High sensitivity in detecting different genotypes. Due to their low cost and easy implementation, they have been widely used in clinical and epidemiological studies	Relatively large size of the PCR fragment, especially in samples that yield poorly amplifiable DNA, such as formalin-fixed, paraffin-embedded materials, or having a high rate of viral integration, loss of target amplification region. Not suitable for type-specific identification	Baleriola et al. (2008)
	Colombia	1810 gynaecology outpatients	38.6	72.2	100			This study
PGMY-LB	Canada	157 participants from other research studies	61.5	95.6	–	Combines PCR assays with type-specific radiolabelled oligonucleotide probes to increase HPV detection sensitivity and reproducibility	Since the β-globin gene is co-amplified with HPV in PGMY assays, these assays could also be more sensitive to the effects of inhibitors	Coutlee et al. (2002)
SPF10-LiPA25	Costa Rica	5659 participants from other research studies	35.3	–	–	System of detection/genotyping capable of amplifying up to 43 different genotypes and providing type-specific genotype information for 25 different HPV genotypes simultaneously	Might have sensibility issues in samples having more than one HPV type because of competition between the different HPV genotypes present in the same and with relative concentrations	Castle et al. (2008)
Amplicor HPV test	France	470 gynaecology outpatients	51.3	96.4	100	Simultaneously detects 13 HR-HPV genotypes with assessment of the presence of the human β-globin gene as positive control	Cross-hybridisation between primers, mainly with HPV56, and requires instrumentation and skilled technologists to extract and perform amplification. Longer time taken to obtain results	Mo et al. (2008)
LINEAR ARRAY HPV genotyping test	United States	5060 females referred to colposcopy	55	89.1	46.5	Detects 37 HPV genotypes individually, including the main 14 carcinogenic HPV genotypes. Uses an internal β-globin control to verify specimen suitability	Lower clinical sensitivity (identification in females with disease). Samples have to be maintained in special media, such as STM, otherwise sensitivity might be affected	Gravitt et al. (2008)
PapilloCheck HPV-Screening Test	France	144 gynaecology outpatients	66.4	–	–	Simultaneously detects and identifies 25 different HPV genotypes, 15 high-risk HPV genotypes (HPV-HR), 2 probably high-risk as well as 8 low-risk (HPV-LR)	Might report detection discrepancies with other methods in multiple infections. Might yield false positives. Detection is directed towards E1 which is more susceptible to DNA sequence modifications (partial deletions) compared to the L1 region	Dalstein et al. (2009)

Abbreviations: SPF: short PCR fragment; LiPA: reverse hybridisation line probe assay.

seems to be affected by competition between the different HPV types present in the same sample, which could thus lead to underestimating HPV genotype prevalence (Table 5) (van Doorn et al., 2006).

The data reported in this study support using GP5+/6+ and MY09/11 as a highly sensitive tool for the PCR detection of HPV infections and coinfections. Such generic viral identification systems can be easily implemented and have a moderate cost. When used together (to avoid underestimating viral infections), they have a wide detection range enabling the identification of more HPV-infected females and have better robustness for determining the epidemiological profiles of HPV types, especially in regions having cervical cancer-associated high morbidity and mortality rates. These profiles would allow a more rational design of screening programs aimed at broader coverage of the most vulnerable populations, especially those having limited access to health services and cutting-edge technology, in turn promoting increased cervical cancer-associated death rates.

Conflict of interest

All the authors involved in this study declare having no conflicting or dual interests.

Role of the funding source

We would like to extend our sincerest gratitude to the Asociacion Investigacion Solidaria SADAR, Caja Navarra (Navarra, Spain) and the Agencia Española de Cooperación Internacional para el Desarrollo (Project 08-CAP2-0609) for financing this project. Study sponsors were not involved in the design, collection, analysis or interpretation of data, nor in the writing of this manuscript or the decision to submit for publication.

Acknowledgments

We would like to thank all the gynaecologists and chief nurses at the health centers involved in this project. We would like to thank Ana Obando and Daniel Leon for their technical support and Jason Garry for revising this manuscript.

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CAPÍTULO 4

“The diagnostic performance of classical molecular tests used for detecting human papillomavirus”

Journal of Virological Methods 185 (2012) 32–38

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

The diagnostic performance of classical molecular tests used for detecting human papillomavirus

Marina Munoz[a,b], Milena Camargo[a,b], Sara C. Soto-De Leon[a,b], Adriana Rojas-Villarraga[b,d], Ricardo Sanchez[a,c], Camilo Jaimes[b], Antonio Perez-Prados[e], Manuel E. Patarroyo[a,c], Manuel A. Patarroyo[a,b,*]

[a] Fundación Instituto de Inmunología de Colombia (FIDIC), Bogotá, Colombia
[b] School of Medicine and Health Sciences, Universidad del Rosario, Bogotá, Colombia
[c] Universidad Nacional de Colombia, Bogotá, Colombia
[d] Center for Autoimmune Diseases Research (CREA), Bogotá, Colombia
[e] Universidad Pública de Navarra, Pamplona, Spain

Article history:
Received 28 February 2012
Received in revised form 19 May 2012
Accepted 24 May 2012
Available online 1 June 2012

Keywords:
Human papillomavirus
Hybrid capture assay 2
Polymerase chain reaction
Screening
Test performance

A B S T R A C T

Cervical samples were evaluated for human papillomavirus (HPV) presence using the hybrid capture-2 (HC2) assay and the polymerase chain reaction (PCR) with three different primer sets (GP5+/6+, MY09/11 and pU1M/2R). PCR results were compared to HC2 and results of all assays were compared to cytological and colposcopy findings. Post-test probability was assessed in individual assays and test combinations. HPV-DNA prevalence was 36.5% with HC2 and 55.2% with PCR. MY09/11 detected HPV-DNA in 38% of samples, GP5+/6+ in 19.1% and pU1M/2R in 16.4%. pU1M/2R and HC2 had the highest concordance (75.31%, $k = 0.39$ in the whole population; 74.1%, $k = 0.5$ in women with abnormal cytology). pU1M/2R had the best diagnostic performance, including optimal post-test probabilities and cervical abnormality detection (individually or in a panel of tests). Women positive for pU1M/2R may be at higher risk of disease progression; the assay performance when combined with a Pap smear in cervical cancer screening programs should be evaluated.

1. Introduction

Cervical cancer is the second oncological cause of death in women worldwide (Bosch and de Sanjose, 2002); as 85% of new cases occur in developing countries where access to screening programs is limited then this preventable disease constitutes a major public health issue (Franco et al., 2003). There are 36.4 cases/year/100,000 women age standardized incidence in Colombia associated with 18.2 cases/year/100,000 women age standardized mortality according to the latest report issued by the World Health Organization in association with the Institut Català d'Oncologia (WHO/ICO, Summary Report 2010).

The main risk factor associated with development of cervical cancer is persistent infection with a high risk human papillomavirus (HR-HPV) altering the physiological cell cycle control and promoting abnormal proliferation of affected cells (Schlecht et al., 2001). Even though HPV infection is a necessary cause of cervical cancer (Girianelli et al., 2009), many other risk factors are involved, such as the life-style of individuals and populations, sexual practices and intrinsic genetic characteristics; these have also been studied widely (Castellsague et al., 2002).

Most women (±90%) who become infected with HPV clear the infection spontaneously within a two-year period; however, a small number will develop cellular atypia but most will experience regression of the lesions requiring no medical intervention and only a small percentage will suffer persistent infection leading to malignant transformation (Moscicki et al., 2006). The Pap smear test is used widely for detecting such cervical abnormalities and, in spite of the high false negative rate and the fact that cellular changes are detected during late stages of infection, this method has proven to be effective in reducing the burden of cervical cancer-derived disease (Nanda et al., 2000). Many molecular biology techniques have become available for clinical and research purposes in response to the need for identifying infection during earlier stages and improving patient follow-up (Molijn et al., 2005).

* Corresponding author at: Fundación Instituto de Inmunología de Colombia (FIDIC), Cra 50 # 26-20, Bogotá, Colombia. Tel.: +57 13244672x141; fax: +57 14815269.
E-mail addresses: marina_munoz@fidic.org.com (M. Munoz), milena_camargo@fidic.org.co (M. Camargo), sara_soto@fidic.org.co (S.C. Soto-De Leon), adrirojas@gmail.com (A. Rojas-Villarraga), rsanchezpe@unal.edu.co (R. Sanchez), camilojaimes12@gmail.com (C. Jaimes), aperez@unavarra.es (A. Perez-Prados), mepatarr@gmail.com (M.E. Patarroyo), mapatarr.fidic@gmail.com (M.A. Patarroyo).

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http://dx.doi.org/10.1016/j.jviromet.2012.05.023

Hybrid capture-II (HC2; Digene, Gaithersburg, MD, USA) is a molecular technique which has been approved by the US Food and Drug Administration (FDA) for DNA-HPV identification; it uses RNA-labeled probes for targeting DNA sequences from 13 HR-HPV and 5 low-risk types (Vernick and Steigman, 2003). Although its use has been widespread in the medical field, it has several limitations including the inability to identify specific types and involves the possibility of crossed-reaction between two sets of probes (Poljak et al., 1999). By contrast, polymerase chain reaction (PCR) is a highly sensitive and specific technique which detects viral DNA even when sample substrate is scarce. The limitations associated with PCR-based HPV-DNA detection are related to primer selection and optimal protocol standardization (Iftner and Villa, 2003). Additionally, the PCR assay can detect residual HPV-DNA from recently resolved infections where cytology is normal; this lowers the sensitivity level for assessing which patients will develop cervical cancer.

The present study was aimed at comparing the performance of different molecular methods for detecting HPV-DNA in cervical samples: HC2 (reference test for HPV-DNA infection) and PCR. Three different primer sets were used for the latter; they were directed towards two viral regions whose integrity is differentially affected during viral integration into the host cell genome. All molecular tests were compared with cytological and colposcopy results for the same women as reference tests for assessing cervical conditions.

2. Materials and methods

2.1. Patient characteristics

A total of 400 women were considered for this study; their voluntary attendance at four Colombian medical centers involved taking a routine cytology test between August 2007 and December 2008. Three of these centers were located in the capital city (Bogota): (i) "Fontibón" ($n = 47$), (ii) "Bosa" ($n = 115$), (iii) "Engativa" ($n = 105$); the fourth center was located in a popular tourist destination near Bogotá, (iv) "Girardot" ($n = 133$).

2.2. Ethical approval

This study was approved by the pertinent ethics committees at Fundación Instituto de Inmunología de Colombia, Hospital Fontibón, Hospital Pablo VI in Bosa, the Hospital de Engativá and the Nuevo Hospital San Rafael in Girardot. Prior to sample testing, all the women received information about the purpose of the study, as well as the risks and benefits associated with it. They then signed a written informed consent form and completed a questionnaire so that sociodemographic data and the risk factors of interest could be obtained. After the samples had been processed, the official results were sent to all the institutions participating in this study and included in the patients' medical records, thereby providing valuable information for the primary care doctor.

2.3. Collection of cervical cells

The Pap smear samples were collected and processed following official Colombian health service guidelines. Women whose samples showed any degree of abnormality were given a colposcopy examination, following the recommendations and procedures laid down in the mandatory Colombian unified health plan (Fig. 1). The Bethesda system was used for reporting the cytological and colposcopy findings (Solomon et al., 2002), and all cases were reviewed by an experienced gynecological pathologist.

2.4. Human papillomavirus DNA detection

Simultaneously, cervical samples were taken for both HPV-PCR and HC2; 95% ethanol (Lema et al., 2001) and Specimen Transport Medium (STM; Digene, Gaithersburg, MD, USA) were used as means of preservation and transport, respectively. An independent entity (Laboratorio-SIPLAS, Sociedad Interdisciplinaria para la Salud) did the HC2 tests based on commercially validated protocols for detecting high-risk HPV types. Test results from all study sites were masked from researchers until data analysis was completed.

DNA was extracted from epithelial cells found in specimens for PCR using a QuickExtract DNA extraction solution commercial kit (Epicentre, Madison, WI), following the manufacturer's instructions. PCR for human β-globin was carried out on all the samples processed using the GH20 and PC04 primers which amplified a 262 base pair segment; this PCR was used to verify the quality of the DNA (Saiki et al., 1985).

The samples showing human β-globin gene amplification were analyzed using three primer sets (GP5+/6+, MY09/11 and pU1M/2R). PCR amplifications were carried out simultaneously and independently in the standardized conditions reported by previous studies (de Roda Husman et al., 1995; Fujinaga et al., 1991; Gravitt et al., 2000), including measures for preventing contamination. Samples which had amplification products using any of the three primer sets were considered to have had positive PCR results for HPV infection. Positive controls were included in all assays; plasmids containing the HPV-16 L1 gene extracted from transfected Sf21 cells were used in the assays carried out with GP5+/6+ and MY09/11, while known positive samples were used in assays carried out with pU1M/2R. DNase- and RNase-free water were used as PCR negative control in all amplifications.

2.5. Statistical analysis

Descriptive statistics were used for clinical data to summarize the characteristics of the baseline population, including the main clinical and demographic variables. A concordance test (Kappa) estimated the agreement between the results for methods tested in the population as a whole and in different categories, according to the presence of cervical abnormality. Kappa values were classified as follows: 0–0.2 = poor, 0.21–0.4 = slight, 0.41–0.6 = fair, 0.61–0.8 = moderate, 0.81–0.99 = substantial and 1.0 = perfect agreement (Remmerbach et al., 2004).

Independent multivariate logistic regression models were run, including the results for all five methods assessed (i. HC2, ii. PCR, iii. GP5+/6+, iv. MY09/11, v. pU1M/2R) as dependent variables, and cytological or colposcopy findings as independent variables. Odds ratios (OR) and 95% confidence intervals (95% CI) were adjusted to a group of covariables (age, ethnicity, marital status, smoking, age of first intercourse, number of sexual partners, pregnancies, contraceptive methods used and history of sexually transmitted diseases (STDs)), all of them are variables that have been associated with an increased risk of infection, however, correlation does not prove causation. A <0.05 P value was considered significant. The Hosmer–Lemeshow goodness-of-fit test was used for assessing the suitability of the logistic model.

All PCR test results were compared to HC2 to evaluate each assay's performance as a tool for detecting HPV infection; the five molecular assay results were then compared to those obtained by cytology or colposcopy. Sensitivity and specificity data were adjusted as these tests are considered to be an imperfect gold standard, given the sensitivity and specificity values reported in previous studies for each of them (Cox et al., 1995; Kumar et al., 2007; Wu et al., 2005). This adjustment was based on an algorithm assuming conditional independence (Zhou et al., 2008). Predictive values and likelihood ratios were calculated for each comparison.

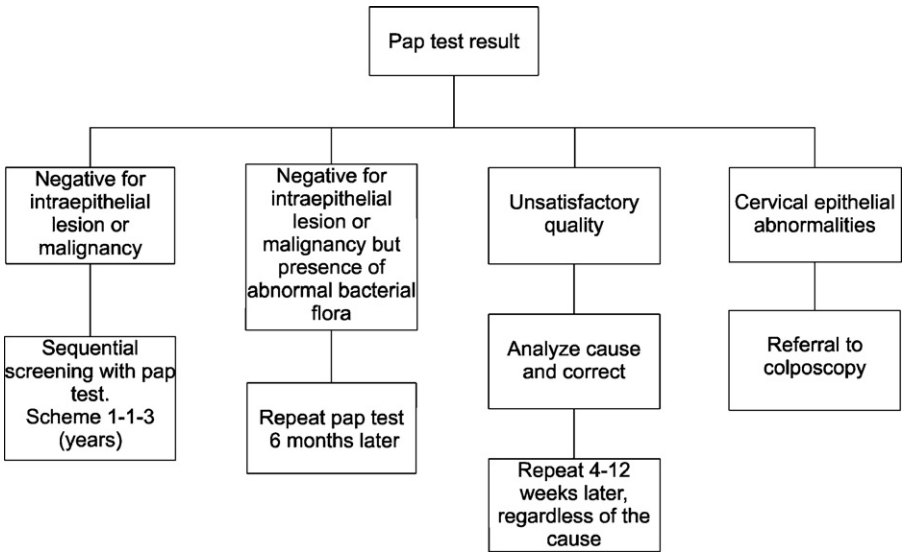

Fig. 1. Screening strategies for cervical cancer detection and control in Colombia. Routine screening according to the guidelines of the cervical cancer detection and control program of the general health security system in Colombia (Posso et al., 2005).

The likelihood ratios for a positive test result (LR+) or a negative test result (LR−) were used to calculate post-test probability, a predictive analysis being made for diagnostic probability using a specific method or combinations of methods. The pre-test probability of HPV infection was compared to the estimated later probability of disease using the information provided by the diagnostic test. The difference between previous probability and later probability was used as a way to analyze the efficiency of the specific diagnostic method or combinations of methods used in parallel. A Diagnostic Test Calculator was used for making the LR calculations (Schwartz, 2002–2007) and STATA 10 software was used for the rest of the analyses.

3. Results

Three of the samples were excluded from statistical analysis due to the incompleteness of the required data. The mean age of the population analyzed ($n = 397$) was 34.7 ± 10.4 years [range: 16–63]. Table 1 shows the distribution of other socio-demographic variables and the risk factors included in this study.

HPV infection prevalence was 55.2% when screening with PCR and 36.5% when using HC2. When discriminating PCR results according to the primer set used, positive readings were found in 19.1% of the samples when using GP5+/6+, 38.0% with MY09/11 and 16.4% with pU1M/2R.

Abnormal cytology findings were detected in 36.0% ($n = 143$) of total samples processed, of which HPV-DNA was detected in 53.8% ($n = 77$) by HC2 and 62.2% ($n = 89$) by PCR; among PCR positive samples, 37.1% ($n = 33$) of them were detected with GP5+/6+, 68.5% ($n = 61$) with MY09/11 and 47.2% ($n = 42$) with pU1M/2R. Of the total number of lesions confirmed by colposcopy ($n = 99$, 24.9%), HC2 detected 56.6% ($n = 56$) and PCR 63.6% ($n = 63$), whereas GP5+/6+ detected HPV-DNA in 30.1% ($n = 19$) of the samples, MY09/11 in 80.9% ($n = 51$) and pU1M/2R in 47.6% ($n = 30$).

A total of 42 (10.6%) samples proved positive in HC2 and negative in PCR, most of them having relative luminescence units (RLU) values ranging from 1.1 to 5 (95.2% ($n = 40$)). Within this subpopulation 17 (40.5%) women also had abnormal cytology, 11 (64.7%) of whom were diagnosed as having some degree of cervical lesion in colposcopy evaluation. Another subgroup

Table 1
Sociodemographic characteristics of the population studied.

Variable (n) category	Individuals per category n (%)[a]
Age (396 women)	
≤20	30 (7.6)
21–30	129 (32.6)
31–40	117 (29.5)
≥41	120 (30.3)
Ethnic background (374)	
White	73 (19.5)
Mestizo	296 (79.2)
African-American	5 (1.3)
Marital status (390)	
Single	90 (23.1)
Married	263 (67.4)
Divorced/widowed	37 (9.5)
Smoker (394)	
No	303 (76.9)
Yes	91(23.1)
Age of first intercourse (396)	
≤16	146 (36.9)
17–19	160 (40.4)
≥20	90 (22.7)
Sexual partners ($n = 390$)	
1	133 (34.1)
2–3	196 (50.3)
≥3	61 (15.6)
Pregnancies (394)	
None	33 (8.4)
1–2	177 (44.9)
≥3	184 (46.7)
Contraceptive methods (380)	
No method used	120 (31.6)
Hormonal	72 (18.9)
Other	188 (49.5)
STD (380)	
Yes	62 (16.3)
No	318 (83.7)

[a] Categories had less than 397, given that data was missing from the surveys. STDs: history of sexually transmitted disease.

Table 2
Degree of cervical abnormality according to cytology and colposcopy results.

Diagnostic test	Total, *n* (%)[a]	ASCUS, *n* (%)[b]	LSIL, *n* (%)[b]	HSIL, *n* (%)[b]
Cytology	368 (92.7)	90 (24.5)	46 (12.5)	7 (1.9)
Colposcopy	196 (49.4)	–	95 (48.5)	4 (2.0)

[a] Percentage of individuals having a result recorded for each test in the total population ($n = 397$).
[b] Rate of subjects having positive test from the total of individuals with the result for each particular technique. – Abnormality not detectable by colposcopy, ASCUS: atypical squamous cells of undetermined significance, HSIL: high-grade squamous intraepithelial lesions, LSIL: low-grade squamous intraepithelial lesions.

consisted of 116 individuals testing positive in PCR and negative in HC2 (29.2%), 25.0% ($n = 29$) of whom had abnormal cytology and more than half of them (62.1%, $n = 18$) had abnormal findings by colposcopy.

Table 2 shows cervical cytology and colposcopy results based on the degree of abnormality. A group of 37 women (9.3%) had negative results for all molecular tests, in spite of having abnormal findings in cytology; 67.6% ($n = 25$) of these women had readings consistent with atypical squamous cells of undetermined significance, 29.7% ($n = 11$) with low-grade squamous intraepithelial lesion and 2.7% ($n = 1$) with high-grade squamous intraepithelial lesion. All of them underwent colposcopy and lesions were identified in 67.5% ($n = 25$) of the cases, including a patient reported as having high-grade squamous intraepithelial lesion.

Overall agreement between HC2 and PCR was estimated to be 60.2% ($k = 0.2$) and the tests having the highest agreement were PCR using pU1M/2R and the HC2 assay (75.3%, k: 0.4). The correlation between the rest of the tests used was poor (see Supplementary file 1). An even higher agreement between HC2 and PCR was observed when cytology and colposcopy results were categorized as being normal/abnormal, having 67.8% (k: 0.3) and 70.7% (k: 0.4) agreement rates, respectively. The pU1M/2R primer set had the highest correlation with HC2 results among the individual PCR assays (74.1%, k: 0.5 for abnormal cytology and 71.7%, k: 0.5 for colposcopy-proven lesion).

Table 3 shows the ORs (adjusted for risk factors, as described previously) showing the association between tests and lesion severity in cytology (A) and colposcopy (B). An additional statistical analysis was run to estimate the ORs, cytology being recorded as either normal or abnormal. These results showed that the association between the pU1M/2R primer set (5.2-CI: 2.4–10.9) and the HC2 assay (2.9-CI: 1.7–5.0) was still significant, whereas colposcopy was only significantly associated with HC2 (2.5-CI: 1.2–5.8) (data not shown).

Table 4 shows the features for each assay regarding its promise as a diagnostic test (sensitivity, specificity, predictive values and likelihood ratios) based on the prevalence obtained from results yielded by techniques considered as standard (HC2 for HPV infection and cytology or colposcopy for cervical abnormalities). Table 5 shows the post-test probabilities for single tests and combinations of tests using the prevalence reported by reference tests found in the pertinent literature.

4. Discussion

Prior studies conducted all around the globe have reported HPV-DNA prevalence determined by PCR, ranging from 5% in certain European regions (de Sanjose et al., 2007) to 50% in some Latin-American countries (Tabora et al., 2009); such significant variation could be related to intrinsic population characteristics and the screening methods used (Bosch and de Sanjose, 2003). Previous research conducted in Colombia using a single primer set (GP5+/6+) to estimate HPV-DNA prevalence in patients having normal cytology has reported positive findings in 14.8% of its sample (Molano et al., 2002). 49.2% prevalence was detected later in cytology samples during 2009 using two different primers sets (GP5+/6+ and MY09/11) (Soto-De Leon et al., 2009), similar to that being reported now using three primers sets (55.2%).

Such relatively higher HPV prevalence could have been the result of the simultaneous use of primers targeting two viral genome regions: (i) GP5+/6+ (Remmerbach et al., 2004) and MY09/11(Qu et al., 1997) both directed towards the L1 late protein promoter and (ii) pU1M/2R, directed at the E6–E7 genes (Fujinaga et al., 1991). This approach was based on published data suggesting that the viral genome undergoes changes during infection thereby allowing it to become integrated into the genome host cell, resulting in total or partial loss of DNA segments. The only regions consistently found to remain intact through the whole microorganism life-cycle are the long control region and E6–E7 oncogenes (Rauber et al., 2008), supporting their use as targets for molecular screening techniques, even in patients having active infection where integration may have occurred already.

The percentage of HPV-DNA detection using HC2 (36.5%) was higher than that usually reported for European countries where prevalence is close to 10% (Giorgi Rossi et al., 2010) and was lower than that for Brazil where HPV-DNA has been detected in 44.9% of samples (Carestiato et al., 2006). Data obtained in the present study using the above test as reference was still high, being consistent with data from other Latin-American countries where population demographics and screening characteristics are similar. Furthermore, the observations indicated that PCR was able to detect viral DNA in more patients compared to HC2, especially in women having abnormal cytology (62.2% and 53.8%, respectively). The above arguments support the previously suggested notion that the

Table 3
Multivariate logistic regression analysis showing the association between test result and cervical lesion severity.

A.	Cytology ($n = 369$), OR (95% CI)				
	GP5+/6+	MY09/11	pU1M/2R	PCR	HC2
ASCUS	1.6 (0.8–3.1)	1.19 (0.7–2.1)	5.09* (2.2–11.8)	1.45 (0.8–2.6)	2.57* (1.4–4.8)
LSIL	0.42 (0.1–1.2)	1.75 (0.8–3.7)	4.65* (1.7–12.5)	1.45 (0.7–3.1)	2.94* (1.3–6.5)
HSIL	–	–	48.61* (6.2–381.1)	1.04 (0.2–5.8)	30.12* (2.8–324.5)
B.	Colposcopy ($n = 196$), OR (95% CI)				
	GP5+/6+	MY09/11	pU1M/2R	PCR	HC2
LSIL	0.4 (0.2–0.9)	0.9 (0.4–1.8)	1.7 (0.7–4.0)	0.3 (0.1–0.7)	2.5* (1.6–5.2)
HSIL	0.6 (0.5–6.8)	0.3 (0.0–4.3)	8.0* (0.7–89.7)	0.2 (0.0–1.9)	4.4 (0.7–54.2)

Odd ratios (OR) adjusted for the variables included in Table 1. A. Lesion reported in cytology. B. Lesion described in colposcopy. – The estimator could not be calculated because one of the fields contained no data during the dispersion analysis. ASCUS: Atypical squamous cells of undetermined significance, CI: confidence interval, HSIL: high-grade squamous intraepithelial lesions, LSIL: low-grade squamous intraepithelial lesions. For detailed information on the raw data and OR calculations, see Supplementary file 2.
* Values having $P < 0.05$.

Table 4
Characteristics for each molecular test as a diagnostic tool compared to references test results.

Test	Reference test	Sensitivity % [95% CI]	Specificity % [95% CI]	PPV [95% CI]	NPV [95% CI]	LR (+) [95% CI]	LR (−) [95% CI]
GP5+/6+	HC2	19.2 [11.8–28.1]	80.9 [71.9–88.2]	53.9 [42.1–65.5]	67.6 [62.2–72.7]	2.0 [1.4–3.0]	0.8 [0.7–0.9]
	Cytology	20.1 [12.7–28.2]	79.9 [70.8–87.3]	44.6 [33.0–56.6]	62.6 [56.8–68.1]	1.3 [0.8–1.9]	0.9 [0.8–1.0]
	Colposcopy	26.1[17.7–35.7]	73.8 [64.3–82.3]	37.3 [24.1–51.9]	44.8 [36.6–53.3]	0.6 [0.4–0.9]	1.2 [1.0–1.4]
MY09/11	HC2	38.1 [28.5–51.9]	62.0 [51.8–71.5]	41.7 [33.8–50.0]	66.7 [60.4–72.5]	1.2 [1.0–1.6]	0.9 [0.7–1.0]
	Cytology	39.4 [29.4–47.3]	60.6 [50.7–70.6]	42.1 [33.9–50.5]	63.2 [56.5–69.6]	1.1 [0.9–1.5]	0.9 [0.8–1.1]
	Colposcopy	52.3 [41.8–62.1]	47.7 [37.9–58.2]	49.5 [39.5–59.5]	48.4 [37.9–59.0]	1.0 [0.7–1.2]	1.0 [0.8–1.4]
pU1M/2R	HC2	16.5 [9.43–24.7]	83.7 [75.3–90.6]	86.2 [75.3–93.5]	73.2 [68.1–77.9]	11.0 [5.5–21.0]	0.6 [0.6–0.7]
	Cytology	16.1 [13.8–18.4]	84.0 [81.6–86.2]	71.2 [57.9–82.2]	67.3 [61.8–72.5]	3.9 [2.3–6.6]	0.8 [0.7–0.8]
	Colposcopy	22.6 [14.3–31.4]	77.5 [68.6–85.7]	66.7 [51.0–80.0]	54.3 [46.0–62.4]	2.0 [1.1–3.4]	0.8 [0.7–1.0]
PCR	HC2	55.3 [44.7–65.0]	44.9 [35.0–55.3]	47.0 [40.3–53.9]	76.4 [69.5–82.4]	1.5 [1.3–1.8]	0.5 [0.4–0.7]
	Cytology	56.0 [45.7–65.9]	44.0 [34.1–54.3]	43.2 [36.3–50.3]	66.7 [58.8–73.9]	1.2 [1.0–1.4]	0.8 [0.6–1.0]
	Colposcopy	72.3 [62.1–80.5]	27.6 [14.5–37.9]	44.4 [36.0–52.9]	33.3 [21.1–47.5]	0.8 [0.6–0.9]	2.0 [1.2–3.2]
HC2	Cytology	36.8 [25.6–47.2]	63.4 [52.8–77.4]	57.0 [48.2–65.5]	71.7 [65.4–77.4]	2.1 [1.6–2.7]	0.6 [0.5–0.7]
	Colposcopy	43.7 [33.1–53.3]	56.5 [45.7–65.9]	65.9 [54.8–75.8]	61.3 [51.5–70.4]	1.9 [1.3–2.7]	0.6 [0.5–0.8]

The molecular test results with the prevalence yielded by reference tests, HC2 for HPV infection and cytology-colposcopy for cervical abnormalities were compared for doing these calculations. CI: Confidence interval, HC2: Hybrid capture-II assay, LR: likelihood ratio, NPV: negative predictive value, PPV: positive predictive value. All calculations regarding the LR are included in the Supplementary file 3 (HC2/cytology/colposcopy).

difference in prevalence data yielded by these techniques could have been due to HC2 failing to detect viral DNA in the specific subset of infected patients having low amounts of HPV-DNA (Jastania et al., 2006).

Regarding cytology reports, it was observed that the prevalence of low- and high-grade squamous intraepithelial lesions was higher than that reported in previous studies from around the world; however, other studies carried out in Colombia have found a high prevalence of cervical abnormality (Uribe et al., 2006). This could have been related to low cervical cancer prevention and control program coverage regarding the Colombian population, meaning that activities aimed at identifying women at greater risk of developing this disease should become intensified. The fact that a group of women had negative molecular results and cervical abnormalities emphasizes the important role of cervical cytology as an effective screening strategy in reducing the burden of cervical cancer. In spite of this, the data has also revealed limitations in detecting HPV-infected women when using just Pap smears, thereby indicating that adding molecular techniques to screening regimes would be of great use.

Agreement was low between the techniques used here, similar to that reported in other studies (Castle et al., 2003); this may have been related to the fact that they target different viral genome elements. The highest concordance was found between HC2 and pU1M/2R which target early genome regions, while the other primer sets were aimed at annealing within the L1 protein promoter. Agreement between tests increased when they were tested in the population suffering cervical abnormalities. This could be explained by spectrum bias or have been related to the hypothesis proposed by previous field research stating that low agreement regarding the results for women having normal smears may have been due to fewer viral copies being present during early disease stages (Munoz et al., 2006), thereby decreasing the ability of HC2 to detect DNA since it is a technique based on signal amplification as opposed to PCR assays which amplify target-specific DNA sequences.

A significant association between molecular tests and cervical abnormality was found, showing that pU1M/2R and HC2 had high odds of detecting women with low-grade squamous intraepithelial lesion compared to the normal group; the likelihood of detecting women with high-grade squamous intraepithelial lesion increased by up to 5 times (consistent with colposcopy and cytology results). This could have been related to the fact that they both recognized early viral genes (E6–E7) considered to play a critical role in malignant transformation secondary to HPV infection (Lorincz, 1996).

Analyzing post-test probabilities for combinations of techniques showed that pU1M/2R had the best diagnostic features and performance as it had a very strong association with a confirmatory HPV infection result and cervical abnormality, yielding results superior to those from HC2 and PCR. It is thus suggested that the molecular identification of HPV infection targeting genome regions remaining unchanged during infection is a valid approach; also, this argument can be supported by the findings related to women evaluated by colposcopy where the highest post-test probabilities included negative readings of molecular tests directed towards late viral regions, which presumably become lost during advanced stages. It should be taken into account here that the good analytical sensitivity reported for GP5+/6+ and MY09/11 in other publications

Table 5
Post-test probabilities for test combinations.

Reference test	Pre-test percentage	Post-test percentage for single test (test results)	Post-test percentage for test combinations (test results)		
			Two	Three	Four
HC2	36.5	86.4: pU(+) 23.7: PCR(−)	92.8: pU(+) and GP(+) 23.4: pU(−) and GP(−)	94.1: pU(+), GP(+) and MY(+) 21.0: pU(−), GP(−) and MY(−)	N/A N/A
Cytology	36.0	71.2: pU(+) 28.3: HC(−)	83.8: pU(+) and HC(+) 23.0: pU(−) and HC(−)	86.8: pU(+), GP(+) and HC(+) 21.6: pU(−), HC(−) and MY(−)	88.2: pU(+), GP(+), HC(+) and MY(+) 20.6: pU(−), MY(−), HC(−) and GP(−)
Colposcopy	24.9	66.7: pU(+) 38.7: HC(−)	79.1: pU(+) and HC(+) 34.2: pU(−) and HC(−)	79.9: pU(+), HC(+) and MY(−) 27.8: HC(−), GP(−) and MY(+)	82.5: pU(+), HC(+), GP(−) and MY(−) 22.1: pU(−), HC(−), MY(+) and GP(+)

The table features the test combinations showing the highest positive predictive value, and the results with the lowest negative predictive value. Calculations were made using reference tests as data sources, HC2 for HPV-DNA and cytology-colposcopy for cervical abnormality. N/A, not applicable considering that HC2 is regarded as the reference test for HPV-DNA infection. All calculations can be seen in Supplementary file 3 (Combined, pag. 1 and 2).

should not be mistaken as good clinical performance (Kinney et al., 2010).

Even though the pU1M/2R primer set had greater precision in detecting infection in women having any degree of cervical abnormality, the percentage of positive samples was lower than that found with primer sets directed against late viral regions. Although results obtained with late primer sets would not be as clinically relevant as those yielded by pU1M/2R, it is worth stressing that they might contribute towards gathering knowledge regarding epidemiology and viral biology during infection stages where viral genome integration has not happened. The detection spectra in different viral types were variable for each primer set, which is why it would be advisable to use primer combinations in HPV identification studies.

In conclusion, test performance evaluation showed that the PCR using the pU1M/2R primer set is an optimal screening tool for HPV-DNA detection and its results indicate that it is a more reliable indicator of malignant transformation, when compared to the more frequently used GP5+/6+ and MY09/11 sets; however, these sets have been shown to be useful for monitoring women having persistent HPV infections, remaining as valuable tools for the early prevention of cervical carcinogenesis. This finding has led us to wonder whether including pU1M/2R in cervical cancer screening programs, along with the Pap smear, might provide doctors with information for the early recognition of women at a higher risk of developing cancer. This would also take into consideration that the costs of applying an additional PCR test could be less expensive compared to treatment costs after the disease has developed. Nonetheless, additional longitudinal and diagnostic cost-effectiveness studies are required to assess the diagnostic accuracy of such test during the different stages of the clinical course of HPV infection.

Acknowledgments

We would like to extend our sincerest gratitude to Asociación Investigación Solidaria SADAR, Caja Navarra (CAN) (Navarra, Spain) and Agencia Española de Cooperación Internacional para el Desarrollo (AECID) (Project 08-CAP2-0609) for supporting and financing this project. We would like to extend our sincerest gratitude to the SIPLAS laboratory for the HC2 assays and thank Cindy Lancheros, Rocio Meneses and Duby Botero for their technical support and Jason Garry for revising this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2012.05.023.

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CAPÍTULO 5

“Persistence, clearance and reinfection regarding six high risk human papillomavirus types in Colombian women: a follow-up study”

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

Persistence, clearance and reinfection regarding six high risk human papillomavirus types in Colombian women: a follow-up study

Sara C Soto-De León[1,2], Luisa Del Río-Ospina[1,2], Milena Camargo[1,3], Ricardo Sánchez[4], Darwin A Moreno-Pérez[1,3], Antonio Pérez-Prados[5], Manuel E Patarroyo[1,4] and Manuel A Patarroyo[1,2*]

Abstract

Background: The design of new healthcare schemes which involve using molecular HPV screening means that both persistence and clearance data regarding the most prevalent types of HR-HPV occurring in cities in Colombia must be ascertained.

Methods: This study involved 219 HPV positive women in all of whom 6 types of HR-HPV had been molecularly identified and quantified; they were followed-up for 2 years. The Kaplan-Meier survival function was used for calculating the time taken for the clearance of each type of HPV. The role of a group of independent variables concerning the time taken until clearance was evaluated using a Cox proportional-hazards regression model or parametric (log-logistic) methods when necessary. Regarding viral load, the Wilcoxon rank-sum test was used for measuring the difference of medians for viral load for each type, according to the state of infection (cleared or persistent). The Kruskal-Wallis test was used for evaluating the change in the women's colposcopy findings at the start of follow-up and at the end of it (whether due to clearance or the end of the follow-up period).

Results: It was found that HPV-18 and HPV-31 types had the lowest probability of becoming cleared (1.76 and 2.75 per 100 patients/month rate, respectively). Women from Colombian cities other than Bogotá had a greater probability of being cleared if they had HPV-16 (HR 2.58: 1.51–4.4 95% CI) or HPV-58 (1.79 time ratio: 1.33-2.39 95% CI) infection. Regarding viral load, HPV-45-infected women having 1×10^6 to 9.99×10^9 viral copies had better clearance compared to those having greater viral loads (1.61 time ratio: 1.01-2.57 95% CI). Lower HPV-31 viral load values were associated with this type's persistence and changes in colposcopy findings for HPV-16 gave the worst prognosis in women having low absolute load values.

Conclusions: HPV infection clearance in this study was related to factors such as infection type, viral load and the characteristics of the cities from which the women came. Low viral load values would indicate viral persistence and a worse prognosis regarding a change in colposcopy findings.

Keywords: HR-HPV, Persistence, Clearance time, Colombia, Follow-up study, Viral load

* Correspondence: mapatarr.fidic@gmail.com
[1]Molecular Biology and Immunology Department, Fundación Instituto de Inmunología de Colombia (FIDIC), Carrera 50#26-20, Bogotá, Colombia
[2]School of Medicine and Health Sciences, Universidad del Rosario, Carrera 24#63C-69, Bogotá, Colombia
Full list of author information is available at the end of the article

Background

Persistent infection with high-risk human papillomavirus (HR-HPV) types is the main (but not the only) cause of developing pre-cancerous lesions or cervical cancer [1-3].

Several types of HPV considered oncogenic have been found to be responsible for around 90% of cancer cases worldwide [3], having high HR-HPV type prevalence rates (i.e. HPV-16, −31, −18, −33, −45 and −58) amongst Colombian women [4]. Our group is aware that multiple infections are of great importance for our population, since more than 40% prevalence has been reported in some regions of the country [4].

It has been described that HPV infection represents a transient phenomenon throughout the whole world, leading to high infection prevalence, even though most cases do not produce cervical lesions and those which do, consist of low grade lesions involving spontaneous regression [5]. However, Colombia does have a high cervical cancer incidence rate and high morbidity-mortality values for a type of cancer which is highly preventable [6,7].

Intrinsic viral determinants, such as the infecting viral type or viral load, have been identified as HPV persistence markers [1,8,9]; however, some socio-environmental factors also play an important role in this type of infection and its possible outcomes [5,10].

The present work seeks to describe both viral and host factors which could be intervening in the persistence and clearance of the most common HR-HPV types in Colombia. This study was mainly aimed at providing epidemiological information illustrating how HPV infection can become eliminated in a target population, bearing the state of the infection (single or multiple) and the most prevalent HR-HPV types in mind.

Methods

Study population and ethical considerations

Women eligible for the present study were voluntarily attending cervical screening consultations in three Colombian cities (Chaparral, Girardot and Bogotá) between April 2007 and March 2010.

The present study involved 3 cities: Bogotá the country's capital having an urban population, Chaparral located in the Tolima department is mainly inhabited by mestizos leading a sedentary life style and Girardot in the Cundinamarca department which has become a tourist destination due to its favourable weather and closeness to Bogotá. Data concerning Chaparral and Girardot was combined in a single category called "other city" to ensure a better analysis of women in this study, as both these cities are small, having similar climates and lying at less than 1,000 masl (Bogotá is 2,600 masl).

As inclusion criteria, all women signed a written informed consent form and completed a questionnaire regarding their sociodemographic characteristics, sexual behaviour and risk factor data before undergoing a gynaecological examination and providing a cervical smear sample. The signature of a parent or guardian was required for females younger than 18 years old. Women who stated that they did not intend to move from their home cities for at least 2 years after the study began were included in the follow-up study.

Amongst exclusion criteria considered, women who had negative HPV results, those whose samples had little DNA in them (to ensure that all PCR assays were performed satisfactorily) or no amplification for the *HMBS* gene were not included. Women who were pregnant at base line and who had less than 3 months or more than 9 months until their next visit were also excluded from the study.

The Papanicolaou test was used for analysing samples and HPV–DNA detection; real-time PCR was used for selecting just HPV positive women. This study was supervised and approved by the relevant ethics' committees at Hospital de Engativá Nivel II (in Bogotá), Hospital San Juan Bautista (in Chaparral in the Tolima department) and Nuevo Hospital San Rafael (in Girardot in the Cundinamarca department).

HPV DNA collection, processing and detection by PCR amplification

Cervical samples were collected with a cytobrush and kept in 95% ethanol at 4°C [11]. DNA from these samples was purified using a commercial Quick Extract Solution kit, following the manufacturer's instructions. Samples were homogenised in 200 μL lysis buffer (10 mM Tris–HCl (pH 7.9), 0.45% Nonidet- P-40, 0.45% Tween 20 and 60 mg/mL proteinase K) and incubated for 6 min at 65°C, followed by 2 min at 92°C. Samples were centrifuged at 13,000 rpm for 10 min and the supernatant was removed and stored at 20°C.

Polymerase chain reaction (PCR) for human β-globin was carried out on samples to check DNA integrity using GH20/PC04 primers [12]. Three HPV generic primer sets were used for HPV-DNA detection, as described in previous studies (i.e. GP5+/GP6+, MY09/MY11 and pU1M/2R) [13].

Viral load determined by real-time PCR

Primers and probes

Specific primers for each viral type and for *Homo sapiens* hydroxymethylbilane synthase (HMBS) were synthesised according to a study published by Moberg *et al.*, [14]. The primers described by Moberg *et al.,* amplified the same region for HPV-33 and −58; a new set of primers aimed at the E7 region of these viral types was thus designed. Designing probes for each viral type and HMBS was based on four parallel duplex real-time PCRs per patient, taking

into account the types included in each reaction (Table 1) and support by integrated DNA technologies.

Cloning and sequencing

Processed samples were used as template for PCR (10 μL final volume), containing 0.5 U/μL Mango *Taq* DNA polymerase (Bioline), 1× Mango *Taq* Color reaction Buffer, 2 mM $MgCl_2$, 250 nM dNTPs, 1 mM of each primer and DNase-free water to fulfil the necessary reaction volume. The PCR protocol for each fragment consisted of initial denaturing for 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 20 s at corresponding melting temperature and 30 s at 72°C. A reaction containing DNA-free water was used as negative control. The amplicons so obtained were purified with a Wizard PCR preps kit (Promega), once their quality has been evaluated on 3.25% agarose gel. A TOPO TA cloning kit was used for ligation, followed by transformation in TOP10 *E. coli* cells (Invitrogen). Several clones which grew on selective LB plates with 50 μg/mL kanamycin were incubated in LB broth at 37°C with 250 rpm overnight. Recombinant plasmids were purified using an UltraClean mini plasmid prep kit (MO BIO laboratories, California, USA) and sequenced with an automatic ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, California, USA). Each insert's integrity was checked by aligning the products with the respective theoretical sequenced fragments of each gene using Clustal W software [15].

Real-time PCR

A NanoDrop 2000 (Thermo Scientific NanoDrop Products) was used for quantifying plasmid DNA and the DNA copy numbers were calculated by using the URI Genomics and Sequencing Center web site [16] (Table 1). Standardised RT-PCR assays with 10-fold serial plasmid dilutions (10^{11}-10^{6}copies) gave a standard curve for each viral type and HMBS gene (−3.2 to −3.5 slope values). Samples were tested for HPV-16, HPV-18, HPV-31, HPV-33, HPV-45 and HPV-58. The human HMBS (hydroxymethylbilane synthase) gene was amplified in all samples to verify DNA integrity and calculate viral copy number per cell. PCR involved using a CFX96 Touch Real-Time PCR detection system which can detect 6 different fluorescent dyes; four real-time PCR reactions were carried out per sample, one for detecting HPV-16, a second for HPV-18 and −31, a third for HPV-33 and −45 and a fourth one for HPV-58 and HMBS.

The HPV-16 PCR mix contained 1× reaction buffer, 1.5 mM $MgCl_2$, 250 nM of each dNTP, 250 nM of each primer, 1.5 U/μL MangoTaq Polymerase (Bioline) and 500 nM probe. The second PCR mix for HPV-18 and −31 consisted of 1× reaction buffer, 2 mM $MgCl_2$, 275 nM of each dNTP, 500 nM HPV-18 primers, 250 nM HPV 31 primers, 1.5 U/μL MangoTaq polymerase and 500 nM of each probe. The HPV-33 and −45 PCR mix included 1× reaction buffer, 1.75 mM $MgCl_2$, 275 nM of each dNTP, 250 nM HPV-33 primers, 500 nM HPV-45 primers, 1.5 U/μL MangoTaq polymerase and 500 nM of each probe. The last PCR mix for HMBS and HPV58 contained 1× reaction buffer, 1.87 mM $MgCl_2$, 250 nM of each dNTP, 500 nM of each primer, 1.5 U/μL MangoTaq polymerase, 200 nM HMBS probe and 500 nM HPV 58 probe. All real-time PCR reactions contained 2 μL of the DNA extracted from each

Table 1 Primers and probes used for qPCR

Region	Viral type	Primer sequence 5′ - 3′	*mT**	Plasmid product quantification	qPCR test	Probe	Probe size (bp)	Quencher
E7	HPV-16	AGCTCAGAGGAGGAGGAT	54	$1.43E10^{12}$	Reaction 1	FAM	78	ZEN/Iowa Black FQ
		GGTTACAATATTGTAATGGGCTC						
E1	HPV-18	CATTTTGTGAACAGGCAGAGC	53.7	$1.19E10^{12}$	Reaction 2	Cy5	80	IBRQ
		ACTTGTGCATCATTGTGGACC						
E6	HPV-31	ACGATTCCACAACATAGGAGGA	53.7	$1.35E10^{12}$		HEX	78	ZEN/Iowa Black FQ
		TACACTTGGGTTTCAGTACGAGGT						
E7	HPV-33	ATTAAGTGACAGCTCAGATGA	53.7	$1.86E10^{12}$	Reaction 3	FAM	78	ZEN/Iowa Black FQ
		ACATAAACGAACTGTGGTGTT						
E1	HPV-45	CCATTTGTGAACAGGCAGAGC	53.7	$1.59E10^{12}$		Cy5	76	IBRQ
		CAACACCTGTGCATCATTCTGA						
E7	HPV-58	CGAGGATGAAATAGGCTTGG	53.7	$1.23E10^{12}$	Reaction 4	HEX	109	ZEN/Iowa Black FQ
		ACACAAACGAACCGTGGCGT						
HMBS		GCCTGCAGTTTGAAATCAGTG	53.7	$1.98E10^{12}$		FAM	76	ZEN/Iowa Black FQ
		CGGGACGGGCTTTAGCTA						

HPV Human papillomavirus, *mT* Melting temperature in °C, *FAM* 6-carboxyfluorescein, *Cy5* FluoroLink Mono Reactive Dye Cy5, *HEX* Hexachlorofluoresceine, *HMBS*, Hydroxymethylbilane synthase.
*Melting temperature for quantitative real-time PCR.

cervical sample and DNA-free water to complete 20 μL volume.

96-well plates were used for each run, including 6 standards for each viral type and HMBS, involving 10-fold plasmid dilutions (10^{11}-10^{6} copy dynamic detection range) and a no template control to rule out DNA contamination.

The thermal cycling conditions for HPV-18, −31, −33, −45, −58 and HMBS consisted of initial denaturing for 5 min at 94°C, followed by 30 amplification cycles for 10 s at 94°C and 30 s at 53.7°C. Initial HPV-16 denaturing was followed by 30 PCR cycles for 30 s at 54°C and 30 s at 94°C.

Viral load values were given as absolute and normalised. The viral load was normalised to cellular DNA input amount, using the following formula: viral load (HPV copies/cell): number of HPV copies/(number of HMBS copies/2) [17].

Statistical analysis

Women who had had both a Pap-smear result and HPV-DNA detected by PCR and who fulfilled the follow-up inclusion criteria (at least 3 follow-ups, leaving 6 to 9 months between visits) were included in the analysis. Women were excluded where the HMBS gene was not amplified by RT-PCR. Analysis was based on type-specific HPV infection rather than on individual women, taking into account that multiple infection is common in the Colombian population [4].

Cox's multivariate regression model was used when calculating sample size; hazard ratios (HR) of at least 2 were thus considered, whenever they had a 5% significance level, 80% power, 0.55 standard deviation of tested covariates and 0.1 correlations between tested covariates. The probability of clearance was set at 0.7, according to previous reports [18,19]. Such suppositions required sample size of at least 86 women. STATA 12 stpower command was used for making the calculations.

Clearance was defined as at least two consecutive type-specific HPV DNA samples proving negative, such samples taken at 6-month intervals following a positive sample [20]. Persistence was defined as the identification of the same HPV type in baseline and follow-up samples [21]. The time taken for HR-HPV infection clearance was calculated in months (95% CI), estimated using the Kaplan-Meier survival function.

The first step was evaluating each variable independently to assess their importance regarding clearance time (Table 2); those variables having a significance level of less than 0.2 in the univariate analysis were included in the multivariable models.

The independent variables included in the multivariable model were city, ethnicity, age of first sexual relationship, number of lifetime sexual partners, family planning method, coinfection and viral load (categorised as low, viral load being lower than 9.99E + 5, middle viral load between 1.00E + 6 to 9.99E + 9 and high viral load being higher than 1.00E + 10) concerning time taken to clearance using a Cox's proportional hazard (PH) regression model or parametric methods (log-logistic) when the PH assumption was violated. The PH assumption was graphically evaluated using log-log plots and a PH test based on weighted residuals using Grambsch and Therneau tests [22]. The choice of parametric model was defined using Akaike information criterion (AIC) and Bayesian information criterion (BIC).

Three categories were assigned to the variable "changes in colposcopy" (alike, improved and worsened) for evaluating changes in colposcopy findings between the results of colposcopy at the start of follow-up and the end of it (whether due to clearance of the virus or not). The Kruskall-Wallis test was used for evaluating the difference in viral load for each viral type and change in colposcopy findings since the sample did not have a normal distribution. The Mann–Whitney test was also used for evaluating viral load according to the state of infection (persistent or cleared).

Categorical variable distribution amongst groups was assessed by Chi-squared test or Fisher's test, as appropriate. Median and interquartile ranges were used for quantitative variables, according to the data distribution. Incidence ratios were estimated using months of follow-up as denominator. $A \leq 0.05$ p value was considered statistically significant; STATA 12 was used for all statistical analysis.

Results

The present work has consolidated data concerning 219 women infected by several HR-HPV types; they became voluntarily incorporated into our follow-up study. All the women included guaranteed to attend a base-line visit and at least 3 follow-up visits with around 6 month difference (±3 months); 23.3% of the population being sampled managed to attend follow-up 4 (i.e. data became available from 5 visits).

Regarding baseline information, 23.7% (n = 52: 18.3-29.9 95% CI) of the women in the study were infected by a single type of HR-HPV (single infection); the rest of the population, 76.3% (n = 167) had multiple infections, distributed as follows: 26% (n = 57: 20.3-32.4 95% CI) had infections having simultaneous detection for 2 types of HR-HPV, 29.7% (n = 65: 23.7-36.2 95% CI) infection by 3 types of HR-HPV, 13.2% (n = 29: 9.1-18.4 95% CI) had positive identification for 4 viral types, 5% (n = 11: 2.5-8.8 95% CI) were infected by 5 high risk types and 2.3% (n = 5: 1–5.2 95% CI) had viral DNA identification for 6 types of HR-HPV.

Participants' age ranged from 17 to 71 years-old (SD 10.8, mean 42.2 years). Most of the participating population was mestizo (98.2%; n = 215: 95.4-99.5 95% CI), and from

Table 2 Baseline characteristics' distribution according to HPV infection stage

Baseline characteristics		Single HPV infection n = (%)	Multiple HPV infection n = (%)	Total	p =
Age (y) (n = 219)	<30	10 (31.25)	22 (68.75)	32	0.54
	30-50	31 (22.96)	104 (77.04)	135	
	>50	11 (21.15)	41 (78.85)	52	
Origin (n = 219)	Bogota	21 (30.43)	48 (69.57)	69	0.115
	Other cities	31 (20.67)	119 (79.33)	150	
No. of family members living together (n = 219)	<=4	35 (24.65)	107 (75.35)	142	0.670
	>4	17 (22.08)	60 (77.92)	77	
***Average monthly income (n = 219)**	Minimum	30 (20.98)	113 (79.02)	143	0.243
	>minimum	22 (28.95)	54 (71.05)	76	
Ethnicity (n = 219)	Indigenous	0	1 (100)	1	1
	Mestizo	52 (24.19)	163 (75.81)	215	
	Afro-descendant	0	3 (100)	3	
Marital status (n = 219)	Married	2 (14.29)	12 (85.71)	14	0.849
	Divorced	1 (33.33)	2 (66.67)	3	
	Single	1 (20)	4 (80)	5	
	Living with partner	48 (24.49)	148 (75.51)	196	
	Widow	0	1 (100)	1	
Healthcare scheme affiliation (n = 219)	Contributory	6 (35.3)	11 (64.70)	17	0.244
	Subsidised	46 (22.77)	156 (77.23)	202	
Age on first intercourse (n = 219)	<18	22 (23.66)	71 (76.34)	93	0.979
	≥18	30 (23.81)	96 (76.19)	126	
Lifetime sexual partners (n = 219)	1	25 (24.51)	77 (75.49)	102	0.804
	>1	27 (23.08)	90 (76.92)	117	
Contraceptive method (n = 219)	No method used	25 (27.78)	65 (72.22)	90	0.470
	Surgery	14 (21.21)	52 (78.79)	66	
	Hormonal	3 (13.04)	20 (86.96)	23	
	Barrier	10 (25)	30 (75)	40	
Number of pregnancies (n = 219)	None	2 (50)	2 (50)	4	0.07
	1-2	25 (28.09)	64 (71.91)	89	
	3-4	15 (16.13)	78 (83.87)	93	
	>4	10 (30.3)	23 (69.7)	33	
Abortions (n = 158)	None	23 (28.05)	59 (71.95)	82	0.481
	1	11 (20)	44(80)	55	
	> = 2	4 (19.05)	17 (80.95)	21	
STD (n = 210)	No	37 (22.16)	130 (77.84)	167	0.112
	Yes	15 (34.88)	28 (65.12)	43	
Colposcopy results (n = 202)	L-SIL	14 (29.17)	34 (70.83)	48	0.819
	H-SIL	0	1 (100)	1	
	Negative	37 (24.18)	116 (75.82)	153	
Cytological findings (n = 219)	ASC-US	1 (11.11)	8 (88.89)	9	0.840
	L-SIL	3 (25)	9 (75)	12	
	Negative	48 (24.24)	150 (75.76)	198	

HPV Human papillomavirus, *p*= P value, *STD* Sexually-transmitted diseases, *ASCUS* Atypical squamous cells of undetermined significance, *SIL* Squamous intraepithelial lesions. Some variables had lower values due to loss of data regarding self-completed questionnaires.
*The minimum average monthly income in Colombia is about US$ 300.

the city of Girardot (66.2%; n = 145: 59.6-72.4 95% CI). The population's socio-demographic and sexual behaviour data was described regarding follow-up (base-line), according to the state of the infection (single infection or multiple infections; Table 2).

Survival data was estimated for each type of HR-HPV regardless of single infection or multiple infections and clearance time for each type (Figure 1). A greater clearance occurred for HPV-33-infected women, followed by HPV-16-infected females, whilst fewer events per month occurred for HPV-18- and HPV-31-infected women (Table 3).

Figure 2 clearly shows that HPV-18 infection was the most persistent; infection had not become resolved in 15 women 2 years later, followed by HPV-31 which was present in 4 women having positive identification for

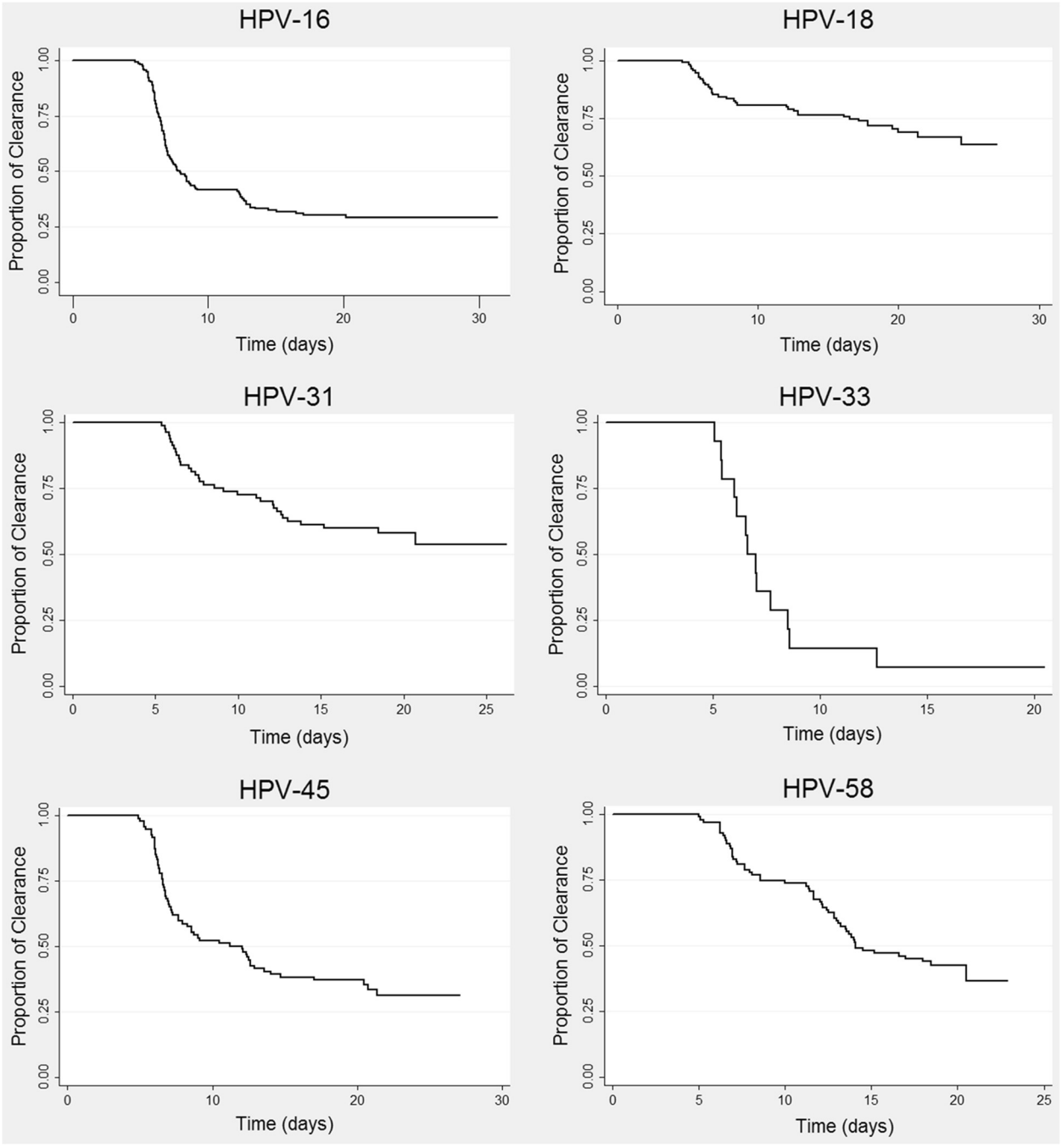

Figure 1 Kaplan-Meier plots of time taken to HPV infection clearance for the 6 HR-HPV types studied here.

Table 3 Prevalence, reinfection and clearance rates concerning the 6 HR-HPV types

Viral type	Baseline			Clearance			Reinfection		
	Prevalence% (n=) [95% CI]	Viral load, median (IQR)		Rate* [95% CI]	Total HPV viral load**, median (IQR)		% (n=) [95% CI]	Viral load, median (IQR)	
		Absolut	Normalised		Absolut	Normalised		Absolut	Normalised
HPV-16	71.2% (156) [64.7-77.1]	2.9E + 6 (9.7E + 6)	0.2 (6.7)	5.9 [4.9-7.2]	2.17E + 09 (7.67E + 10)	5 (13,468)	48.6% (53) [39–58]	2.9E + 6 (9.2E + 6)	0.32 (5.3)
HPV-18	53% (116) [46.1-59.7]	4.3E + 6 (4.56E + 07)	0.42 (13)	1.76 [1.3-2.4]	6.56E + 09 (6.34E + 11)	208 (30,101)	12% (7.4-20.4)	3.0E + 6 (4.28E + 07)	0 .1 (1.8)
HPV-31	37% (81) [30.6-43.8]	9.04E + 07 (6.29E + 09)	44 (3,360)	2.75 [1.96-3.84]	1.23E + 07 (3.02E + 07)	7 (96)	50% (17) [32–67]	1.96E + 09 (7.61E + 09)	3,360 (12,400)
HPV-33	6.4% (14) [3.5-10.5]	2.96E + 07 (1.14E + 09)	3.6 (39,100)	11.51 [6.7-19.8]	1.39E + 9 (1.91E + 10)	774 (4,683)	38.5% (5) [14–68]	1.47E + 08 (5.82E + 08)	51 (128)
HPV-45	43.4% (95) [36.7-50.2]	2.48E + 06 (1.12E + 09)	0.33 (180)	5 [3.9-6.42]	1.84E + 08 (9.30E + 11)	18 (1E + 05)	43.5% (27) [30.9-56.7]	1.14E + 08 (1.94E + 09)	17 (7,180)
HPV-58	45.7% (100) [38.9-52.5]	6.14E + 05 (2.1E + 06)	0.4 (15.7)	4.05 [3.1-5.24]	4.35e + 08 (5.92e + 10)	141 (5,749)	0	0***	0

*Clearance rates are given in values per month, per 100 individuals.
**Total HPV viral load was taken as the mean of the other HPV type infections on clearance.
***The load for other HPV-58 types was not calculated as there was no re-infection.
HPV Human papillomavirus, *IQR* Interquartile range.

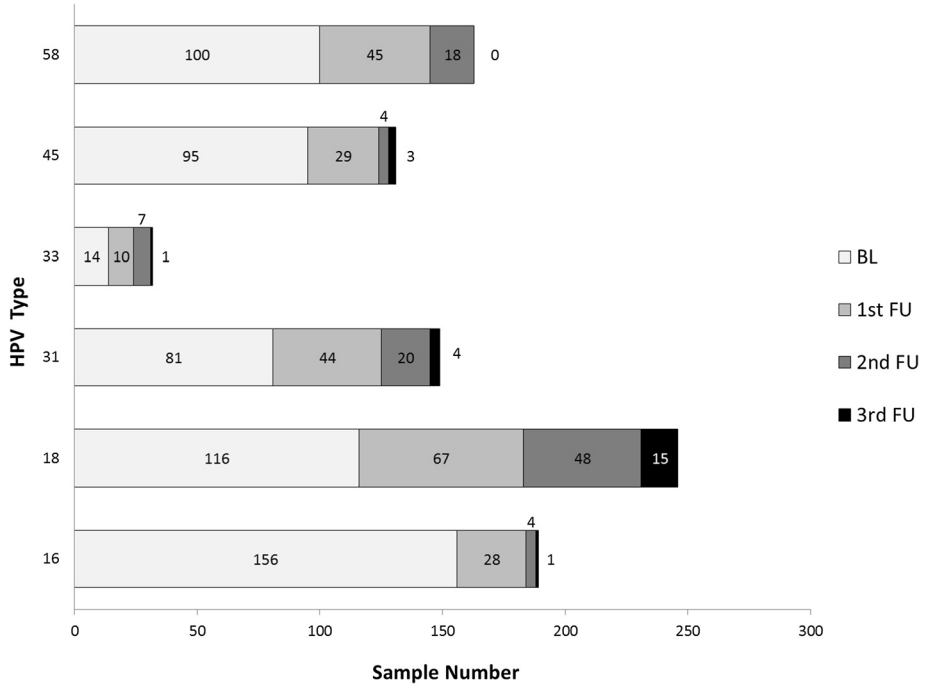

Figure 2 Persistence rates for the 6 HR-HPV types. BL: baseline; 1st FU: first follow-up; 2nd FU: second follow-up; 3rd FU: third follow-up.

this type during each follow-up visit (infection remaining unresolved by the end of the study).

Specific viral load type values were also determined in this study; Table 3 gives both absolute and normalised viral load values for each type infections at the start of the study. It is worth highlighting that those infected by HPV-31 had the highest viral load values, even those normalised by the number of cells, whilst HPV-16 gave the lowest viral load values.

Hazard ratios were calculated for two (HPV-16 and-18) of the 4 most prevalent viral types in molecular determination (i.e. HPV-16, −18, −45, −58), bearing in mind the most important variables in univariate models, for each type (data not shown) (Table 4). Time ratios were calculated for the remaining types, since these (HPV-45 and −58) did not comply with supposed proportional risks for the aforementioned variables; variables could not thus be re-categorised nor could they be assumed to be time-dependent variables. Regression was thereby modelled using a parametric model, bearing in mind the shape of the hazard function and AIC and BIC. The log-logistic model gave the best fit for both types with the foregoing criteria.

Multivariate model values showed that the probability of clearance when a woman had HPV-16 or HPV-58 infection became significantly increased in women from another city compared to women living in the capital. It is worth stating that both types belong to the same species (A9).

Regarding the types belonging to species A7, HPV-18 infection did not have a statistically significant association with any variable evaluated here. However, it was observed that the probability of clearance regarding HPV-45 became significantly increased when the absolute viral load for this type ranged from 1×10^6 to 9.99×10^9 compared to loads equal to or greater than 1×10^{10}.

It should be highlighted that single or multiple HR-HPV infection was not associated with time to clearance in the present sample, since coinfection values for any HPV type were not statistically significant in this model (Table 4).

When evaluating the medians for normalised viral load (per cell) for each type, according to the state of infection at the end of follow-up (cleared or persistent), it was observed that the median for HPV-31 type in the group of women where clearance was found (median = 332.5; IQR = 12,399.72) was greater than the median for those where this virus was not cleared (median = 9.4; IQR = 1,659.98, p = 0.0450). There were no differences in any of the groups regarding the medians for the other HR-HPV types.

The change in colposcopy findings was also evaluated concerning the result at the start of follow-up and the result at the moment of clearance, or at the end of follow-up (Figure 3). There were statistically significant differences regarding HPV-16 concerning absolute values for viral loads for each group, since the value for the group which became worse regarding diagnosis by colposcopy was lower (median = 89,300; IQR = 253,600) than

Table 4 Determinants of clearance for the most prevalent HR-HPV types

Time independent variables		HPV-16	HPV-18	HPV-45	HPV-58
		Multivariable model HR (95% CI)	Multivariable model HR (95% CI)	Multivariable model Tm R (95% CI)	Multivariable model Tm R (95% CI)
Age	**>50**	0.99 (0.53-1.86)	0.59 (0.18-1.87)	1.13 (0.63-2.02)	0.94 (0.63-1.38)
	35-50	1.14 (0.66-1.96)	0.79 (0.35-1.75)	0.77 (0.49-1.20)	1.26 (0.89-1.79)
	<35	*Reference*	*Reference*	*Reference*	*Reference*
City	**Other city**	**2.58 (1.51-4.4)**	1.21 (0.51-2.9)	0.97 (0.62-1.52)	**1.79 (1.33-2.39)**
	Bogota	Reference	Reference	Reference	Reference
Ethnicity	**Afrodescendant**	2.35 (0.69-7.93)	0.85 (0.11-6.61)	0.42 (0.16-1.12)	-
	Mestizo	*Reference*	*Reference*	*Reference*	*Reference*
	Other*	-	-	-	1.17 (0.47-2.89)
Contraceptive method	**No method**	1.01 (0.65-1.59)	0.75 (0.33-1.73)	0.84 (0.56-1.27)	0.98 (0.73-1.30)
	Hormonal	1.45 (0.72-2.96)	1 (0.35 -2.9)	0.76 (0.45-1.26)	0.97 (0.54-1.72)
	Other	*Reference*	*Reference*	*Reference*	*Reference*
Lifetime sexual partners	**1**	1.24 (0.83-1.86)	1.2 (0.61-2.51)	1.05 (0.72-1.52)	1.10 (0.84-1.44)
	>1	*Reference*	*Reference*	*Reference*	*Reference*
Age at first Intercourse	**≥18**	0.93 (0.6-1.41)	1.08 (0.53-2.19)	1.23 (0.85-1.77)	1.13 (0.85-1.51)
	<18	*Reference*	*Reference*	*Reference*	*Reference*
Coinfection	**Yes**	0.87 (0.46-1.65)	2.49 (0.54-11.45)	1.06 (0.46-2.45)	0.87 (0.58-1.32)
	No	*Reference*	*Reference*	*Reference*	*Reference*
Viral Load**	**Low**	2.1 (0.87-5.15)	0.48 (0.08-2.93)	1.27 (0.76-2.12)	1.36 (0.81-2.29)
	Middle	1.49 (0.64-3.51)	0.74 (0.24-2.27)	**1.61 (1.01-2.57)**	1.04 (0.61-1.78)
	High	*Reference*	*Reference*	*Reference*	*Reference*

*Since only 2 women of other ethnicity had HPV-58 (1 Afrodescendant and 1 Indigenous) they were put in a category "Other", just for this type.
**Low viral load meant a viral load below 9.99E + 5, middle 1.00E + 6 to 9.99E + 9 and high meant a viral load higher than 1.00E + 10.
Values in bold = $p < 0.05$.
HPV Human papillomavirus, *CI* Confidence interval, *HR* Hazard ratio, *Tm R* Time ratio.

that for the other groups (improved: median = 2.9×10^6; IQR = 1.1×10^7; alike: median = 2.9×10^6; IQR = 9.9×10^6) ($p = 0.046$). There were no differences in the rest of the types evaluated here regarding viral load according to change in colposcopy findings.

When evaluating normalised viral load according to the degree of HPV infection and colposcopy findings at the start and end of follow-up, it was found that median viral load for HPV-58 was greater for women who had a better prognosis (median = 3.98; IQR = 351.9) compared

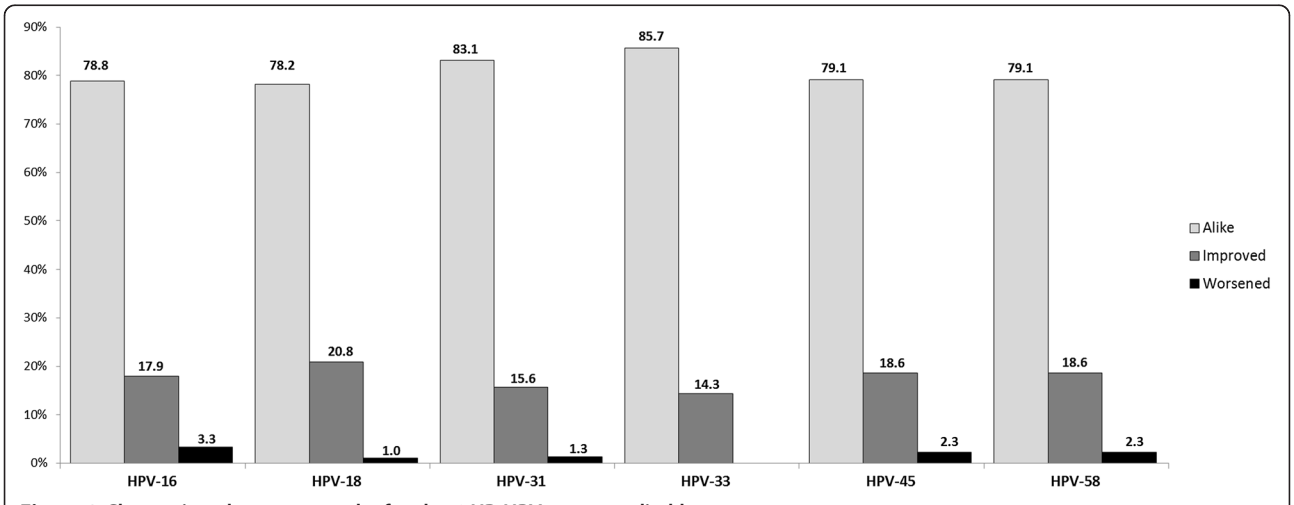

Figure 3 Change in colposcopy results for the 6 HR-HPV types studied here.

to those whose prognosis remained the same (alike) (mean = 0.013; IQR = 0 .68), only in the group of women having persistence for this virus (p = 0.012).

The number of reinfections for each viral type was determined (Table 3); HPV-58 was the only viral type for which there were no reinfection events during the time follow-up lasted.

Discussion

This study has provided detailed epidemiological data for six HR-HPV types present in a Colombian cohort. This has been the first study in Colombia (to the best of our knowledge) aimed at using real time quantification of DNA from the 6 most prevalent types of HR-HPV, giving absolute and normalised load values.

Findings concerning the virus' persistence for the 6 types included here have demonstrated that the risk of acquiring a later HPV infection becomes increased in women already infected by any type of HPV (regardless of complying with a phylogenetic relationship [23], mainly between high risk types [24,25]). Such coinfection could have been the result of immune system deficiency regarding clearance, thereby facilitating viral persistence at the infection site [25].

Regarding the state of infection (single or multiple) and its relationship to clearance time, this work did not reveal an important relationship between such aspects. Various studies have shown that type specific HPV clearance seems to occur regardless of coinfection in an immunocompetent population [26,27].

The highest clearance rates in our cohort were observed for HPV-33 and HPV-16. Previous reports have shown that HPV-16 clears out after other HR-HPV types [26,28,29], but in or study, this type of infection displayed a more transient pattern. Our results are in agreement with previous studies showing that the majority of women with a type-specific infection are negative for that particular viral after one year [19,30].

The reduced clearance rates observed for infections with HPV-18 and −31 types is particularly important bearing in mind that HPV-31 was found in high prevalence in our country [4] and that HPV-18 has been detected in aggressive forms of cancer [31].

Despite HPV-31 was not evaluated in the multivariable model due to the low sample size, it is worth noting that it displayed the highest viral load values and one of the lowest clearance rates. Future studies analysing more women infected with this viral type might help to a better understanding about the influence of viral loads in the type-specific clearance process.

HPV-16, −18 and −58 viral load values did not have a clear relationship with clearance time. This has already been shown for a population from Bogotá in a study involving semi-quantitative identification of viral DNA [24]. The present study showed that, regardless of using a more sensitive technique, no relationship was established between viral load and clearance time for these types, not just in Bogotá, but also in other Colombian cities.

Another factor associated with clearance time was city of origin, showing that women infected by types from the A9 species became cleared more rapidly if they came from Girardot/Chaparral. It is supposed that these cities have factors related to sexual behaviour or cultural characteristics which were not measured in this study and which would have modulated such findings. When analysing the control arm of the large randomised PATRICIA study, it was found that region of origin was one of the behavioural determinants of clearance time, as north-American women took less time to clearance than their European counterparts [20].

Regarding ethnicity, fine control was not used for obtaining it; thus, other ethnic characteristics which were not controlled in this study may have intervened in such marked association between city and clearance time. Another aspect concerned the women's nutritional state; most women were from low socio-economic strata. However, nutritional and/or feeding data was not controlled and may have provided more detailed characteristics concerning the population's idiosyncrasies. Previous studies have shown that women who consumed one or more servings of vegetables per day cleared their HPV infections more quickly than women who did not consume vegetables daily [32]. The intake of lower levels of micronutrients found in vegetables has been associated with increased persistence [33].

Other factor that was not measured in this study but that could influence HPV clearance is hygienic practices. In a cohort of university students, it has been shown that the use of tampons was associated with a reduced rate of HR-HPV clearance [32].

Very interesting data for three A9 species types (HPV-16, −31 and −58) was revealed when determining viral load according to the state of infection and colposcopy findings since these had low viral load values (absolute or per cell) associated with greater lesion severity at the end of follow-up or when infection did not become eliminated. Besides intermediate viral load values for HPV-45 (A7 species) were associated with faster time to clearance, this may be a factor related to transient infection. Such results could have been due to immune system evasion mechanisms since it has been reported that low HPV viral load values have been related to persistent infection [34] and it could be suggested that higher viral load values could be detected efficiently by the immune system and rapidly eliminated.

This is contradictory with studies proposing that high viral loads facilitate persistence, specifically, it has been shown that HPV-16 viral loads in LSIL and HSIL were

higher compared with no intraepithelial lesion or malignancy [35].

This work has several strengths, such as having compiled data from two important focuses of HPV infections (i.e. Girardot and Bogotá), determined viral load using the most sensitive technique for doing so and the percentage of multiple infections revealing an important Colombian populational characteristic. However, the study had difficulties in terms of follow-up times, since infection transience meant that shorter follow-up times than the ones established here may probably have led to obtaining more precise clearance and incidence values. Our information was limited to using prevalent high risk infections for analysing persistence and clearance of infection; the foregoing means that follow-up studies are needed to facilitate understanding the most prevalent epidemiological HPV patterns for Colombia.

Diagnosing HPV infection in clinical specimens has been widely accepted to date in Colombia; viral DNA identification in this type of sample has been included in the Obligatory Healthcare Plan, 2012. The following step must thus be to incorporate monitoring from the identification of HPV infection in cervical cancer control schemes. Such work thus contributes towards the search for a correct algorithm for defining HPV DNA screening since the time taken for most women to clear the virus must be determined for calculating the determinants of such scheme and be referred to regular monitoring [19].

Conclusions

Time to clearance in Colombian females infected by the most frequently occurring HR-HPV types in the sample population was not modulated by infection status (single or multiple). However, viral load played a role in terms of infection regarding HPV-45 and the origin of HPV-16 and −58 infection. Viral persistence and worsening of cytological findings were related to lower HPV-16, −31 and −58 viral loads. All women in our sample who eliminated HPV-58 were not infected again by this viral type. Given that time to clearance was related to lesion development, such information should prove significant when designing HPV DNA primary screening in Colombian healthcare systems, as well as in developing countries.

Abbreviations

HPV: Human papillomavirus; HR-HPV: High risk human papillomavirus; L-SIL: Low squamous intraepithelial lesion; H-SIL: High squamous intraepithelial lesion; ASCUS: Atypical squamous cells of undetermined significance; HMBS: Hydroxymethylbilane synthase; PCR: Polymerase chain reaction; RT-PCR: Real-time PCR; DNA: Deoxyribonucleic acid; STD: Sexually-transmitted diseases; SD: Standard deviation; CI: Confidence interval; HR: Hazard ratio; Tm R: Time ratio; IQR: Interquartile range; AIC: Akaike information criterion; BIC: Bayesian information criterion.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed ideas to this paper and reviewed the manuscript for important intellectual content. SCSDL and MC provided the concept, acquired, analysed and interpreted data, designed the study and wrote the manuscript. LDRO and DAMP developed the methodology and were involved in drafting the manuscript. RS provided statistical analysis, interpreted data and helped in writing the manuscript. The study was supervised by APP, MEP and MAP who provided expertise regarding the discussion of results. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank the hospital San Rafael in Girardot, Hospital San Juan Bautista in Chaparral and Hospital de Engativá in Bogotá, as well as the people who collaborated in obtaining the patients who participated in this study. We would also like to thank Elizabeth Vargas who coordinates the Universidad del Rosario's Cellular and Molecular Biology laboratory for providing us with facilities for developing the methodological part of the study and Jason Garry for translating this manuscript.

Author details

[1]Molecular Biology and Immunology Department, Fundación Instituto de Inmunología de Colombia (FIDIC), Carrera 50#26-20, Bogotá, Colombia. [2]School of Medicine and Health Sciences, Universidad del Rosario, Carrera 24#63C-69, Bogotá, Colombia. [3]Faculty of Natural and Mathematical Sciences, Universidad del Rosario, Carrera 24#63C-69, Bogotá, Colombia. [4]School of Medicine, Universidad Nacional de Colombia, Carrera 45#26-85, Bogotá, Colombia. [5]Mathematics Department, Universidad Pública de Navarra, 31006 Pamplona, Spain.

Received: 2 May 2014 Accepted: 10 July 2014
Published: 16 July 2014

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doi:10.1186/1471-2334-14-395
Cite this article as: Soto-De León *et al.*: Persistence, clearance and reinfection regarding six high risk human papillomavirus types in Colombian women: a follow-up study. *BMC Infectious Diseases* 2014 **14**:395.

CAPÍTULO 6

“Viral load dynamics regarding six types of high-risk human papilloma virus in a cohort of Colombian women”

Viral Load Dynamics Regarding Six Types of High-Risk Human Papilloma Virus in a Cohort of Colombian Women

Sara Cecilia Soto-De León [1,2], Luisa Del Río-Ospina [1,3], Milena Camargo [1,3], Ricardo Sánchez [4], Antonio Pérez-Prados [5], Manuel Elkin Patarroyo [1,4], Manuel Alfonso Patarroyo [1,3*]

[1] Molecular Biology and Immunology Department, Fundación Instituto de Inmunología de Colombia (FIDIC), Carrera 50#26-20, Bogotá, Colombia

[2] Faculty of Natural and Mathematical Sciences, Universidad del Rosario, Carrera 24#63C-69, Bogotá, Colombia

[3] School of Medicine and Health Sciences, Universidad del Rosario, Carrera 24#63C-69, Bogotá, Colombia

[4] School of Medicine, Universidad Nacional de Colombia, Carrera 45#26-85, Bogotá, Colombia

[5] Mathematics Department, Universidad Pública de Navarra, 31006, Pamplona, Spain

* Corresponding author: Professor Manuel A. Patarroyo, M.D., Dr.Sc.

E-mail: mapatarr.fidic@gmail.com (MAP)

Abstract

Human papilloma virus (HPV) infection itself does not predict the development of cervical disease; this means that identifying the viral loads of the HPV types involved in oncogenesis is of interest in discerning the prognosis for these infections.

Data provided by 219 HR-HPV infected Colombian women was analysed; they formed a cohort of women voluntarily attending promotion and prevention controls in 3 sociodemographically diverse cities. All the women included in the study were guaranteed a base-line visit and at least 3 follow-ups (around 6 months apart).

RT-PCR was used for type-specific quantification of the six HR-HPV types occurring with the greatest frequency in this population and the number of viral copies per cell was calculated, identifying the HMBS constitutive gene's load. The analysis of viral loads mean's dynamics over time was performed using generalised estimating equation (GEE) models that included unstructured correlation matrices to account for the autocorrelation between repeated measures.

The viral load for the 6 most common HR-HPV types in Colombia (regarding our target population of older women) was positively linked to factors such as age, ethnic background, family planning method, having had an abortion and viral type.

The women's city of origin for this cohort, their number of sexual partners and concomitance with sexually-transmitted disease different to HIV was related to a reduced viral load for HPV-16, -18 and -45.

Our results led to establishing differential patterns regarding viral load for the most frequently occurring HR-HPV types in the Colombian females.

Introduction

Cervical cancer (CC) is the third type of cancer most commonly affecting women worldwide [1]. The human papilloma virus (HPV) is the necessary agent for pre-carcinogenic lesions and invasive cancer to develop [2].

The types infecting the mucosa have been divided into high- (HR) and low-risk (LR) HPV types, according to their efficiency regarding producing oncogenicity. The persistence of infection caused by HR-HPV types leads to the development of cervical lesions [3].

The identification of the HPV is not in itself evidence of the risk of developing CC; quantifying viral load thus becomes a tool in the diagnosis, prognosis and management of CC at clinical level. Studies have indicated that HPV quantification is a predictor of the risk of developing CC, even before cytological alterations have become evident [4].

Measuring persistence has a prognostic value in understanding the natural history of HPV infection and the development of CC; however, studies directed towards establishing factors influencing the risk of developing CC according the viral load, are required. One important variable concerns HR-HPV types' differential biological behaviour [5].

A study of middle-aged adult women cohorts is important for ascertaining load values and patterns which could have clinical importance, as studies in cohorts of young women have provided important guidelines for managing HPV infection.

This study was aimed at identifying the viral load dynamics of the 6 most frequently occurring HR-HPV types in the Colombian population by quantifying HPV infected patients' viral load in a cohort of adult women having heterogeneous sociodemographic characteristics.

Materials and Methods

Study population

All the women included in this study were voluntarily attending cervical cancer promotion and prevention consultations in three Colombian cities (Chaparral, Girardot and Bogotá). This cohort of patients had been attending consultation over a period covering 2007 to 2010 (3 to 9 month intervals). Todas las mujeres involucradas en el studio, como criterio de inclusion, debieron firmar un consentimiento informado y llenar un cuestionario donde se consignaron los datos sociodemograficos y de factores de riesgo, adicionalmente debían asegurar su permanencia en la ciudad por lo menos los 2 años del estudio.

Los criterios de exclusión fueron ausencia de HPV, muestras que no tuvieran la cantidad suficiente para asegurar todos los resultados de PCR, no amplificación del gen hmbs, mujeres embarazadas en línea de base o que presentaran tiempos entre visitas diferentes a los consignados en el seguimiento (3-9 meses).

Minimum suitable sample size was calculated by using an algorithm designed by Twisk which led to comparing the means of repeated measures between groups [6]; the following assumptions were considered for calculations: Difference between viral copy means of 3.23×10^5 between follow-ups, with a standard deviation for measurements of 2.04×10^6 viral copies, a number of 3 follow-ups with a correlation between them of 0.2, a ratio of 3:1 between groups being compared, having 5% significance value and 80% power. The forgoing assumptions were derived from previous studies [7-9], giving a sample size of 195 women. At least n=146 and n=49 women were required for multiple and single infections, respectively.

Ethics Statement

All the women signed an informed consent form and provided sociodemographic data requested by a questionnaire during each visit; data regarding sexual pattern and risk factors was recorded and a cervical sample taken for cytology and detecting and quantifying HPV.

The data regarding this cohort came from a population included in a prior study [10]; this study was supervised and approved by the following ethics' committees: Hospital de Engativá Ethics Comitee (population in Bogotá), Hospital San Juan Bautista Ethics Comitee (in Chaparral - Tolima department) and Nuevo Hospital San Rafael Ethics Comitee (in Girardot - Cundinamarca department).

Collecting and processing cervical samples and detecting DNA from HPV

The methodology for collecting and processing DNA samples for every woman as well as that for determining viral load has been described previously [10].

El ensayo de PCR en tiempo real se utilizó para la cuantificación de los 6 tipos de HPV incluidos en este studio y el gen constitutivo celular (hmbs). Se estandarizó utilizando diluciones seriadas para cada gen involucrado (10^{11}-10^{6}copies) y se construyó una curva estándar para la posterior cuantificación de las muestras desconocidas. Los ensayos se montaron en dúplex para la cuantificación de HPV-18 and -31, HPV-33 and -45, HPV-58 and HMBS y una única reacción para HPV-16. Las condiciones y secuencias de los primers y sondas están descritas detalladamente en una publicación previa [10].

The amount of DNA from the cervical samples differed widely, thus leading to the need for normalising the HPV load result by the sample's number of cell copies. Normalised viral load was calculated by using the following equation [11]:

$$\text{Number of copies of HPV/cell} = \frac{\text{Number of copies HPV}}{\text{Number of copies HMBS/2}}$$

Statistical analysis

The data for this study involved compiling survey information regarding sociodemographic data and sexual pattern provided during each visit. Normalised viral load values were calculated during each visit and expressed on logarithmic scale.

The data was analysed separately for each viral type since infection by a determined type of HPV not excluding infection by another type, i.e. a woman provided her data several times as infection by different viral types.

Categorical variable distribution between groups was determined according to the viral type detected in the base line visit sample (Visit 1). Such data was presented in terms of frequencies and percentages along with their corresponding confidence intervals within the sample being studied.

Bearing in mind the non-normal distribution of viral load for each type, these have been represented in Figure 1 as the mean of absolute load values for each visit.

The generalised estimating equations (GEE) method was used on the women's follow-up data organized in a panel for analysing the viral load values for different viral types bearing in mind the data's longitudinal origin and N repeated measures for the dependent variable. Infection by each HR-HPV type was treated in this regression model as an independent outcome, meaning that a model had to be made for each viral type.

It was taken into account that the dependent variable was continuous and had non-normal distribution to ensure that the GEE model was suitable, thereby modelling the logarithm for normalised viral load. The model led to coefficients of correlation being obtained, representing the expected change in the log of the mean for viral loads by each change in each covariable. Dependent variable (viral load) auto-correlation structure was also determined for each type by preliminary evaluation of repeated measures correlation matrices. It was found that a non-structured correlation matrix was the most efficient method for adjusting the data, since there was no auto-regressive or independence pattern.

Each type's viral load was proposed as dependent variable for such analysis and those whose coefficients of correlation were significant in the bivariate model as independent variables, such as origin, age, race, age on first sexual relation, the number of sexual partners, family planning method, the number of children, having a background of abortions, sexually-transmitted diseases (STD) and infection by more than one HPV type.

Change in normalised viral load [10] (expressed in logarithmic units) for each type in this study was evaluated by correlating the presence of co-variables which might have represented risk factors; the values were interpreted according to the coefficients resulting from the model. Viral type HPV-33 was not included in the model due to reduced sample size. STATA 12® was used for all statistical analysis.

Results

This work consolidated the data provided by 219 HR-HPV-infected women who became voluntarily incorporated into our follow-up study. Given the availability of patients and maintaining a 3:1 ratio, n=167 women suffering multiple infections and

n=52 having a single infection were included in the study. Women were excluded whose samples were insufficient for analysis and negative for HMBS. All the women who became included were guaranteed a base line visit and at least 3 follow-ups (around 6 months apart: $\pm$ 3 months); 23.3% of the population sampled here managed to attend 4 follow-up visits, i.e. providing information from 5 visits.

This was a cohort of mature women since participants' mean age was 42.2 years-old (10.8 years standard deviation (SD)). The population's sociodemographic and sexual pattern data were described for the start of follow-up (base line), according to the infecting viral type found (Table 1).

Viral types in the population were distributed as follows: HPV-16: 71.2 % (n=156, 95%CI), HPV-18: 53% (n=114, 95%CI), HPV-31: 37% (n=81, 95%CI), HPV-33: 6.4% (n=14, 95%CI), HPV-45: 43.4% (n=93, 95%CI) and HPV-58: 45.7% (n=100, 95%CI).

The graph in Figure 1 was drawn according to the means of viral loads found during each visit. It can be seen that HPV-31 and -33 types began with the highest absolute values and the mean of their load rapidly fell to 0; HPV-16 viral type (in spite of having rapid clearance), reappeared with mean load values (10^6–10^9 viral copies) during visit 5 of the women who managed to continue their follow-up. HPV-18 was the only viral type from this cohort having mean load different to zero after the first follow-up, having similar mean load values during the first three follow-ups.

It was found that coming from a city different to Bogotá was involved in reducing normalised load for HPV-16, -18 and -45 types when running the logistic regression model from generalised estimated equations for analysing factors predicting an increase in viral load. The most significant value was found for HPV-18 type, since the value of

its viral load became reduced by almost 1 unit on the logarithmic scale (coef: - 0.91: - 1.44 to - 0.38 95%CI; p=0.001) for women from Girardot and Chaparral (Table 2).

HPV-31 was the single type which was correlated with age, having an increase of 0.05 logarithmic units as HPV-31-infected women's age increased by 1 year (0.02 to 0.08 95%CI; p=0.001).

Regarding ethnic background, white women had a significant increase in HPV-18 and HPV-58 viral load, since load values increased 2.16 and 4.82 logarithmic units, respectively.

Interestingly, a factor negatively modulating viral load was the number of sexual partners. HPV-18-infected women in this cohort who had had more than 2 or 2 sexual partners during their lives had a reduction of 0.69 logarithmic units and those with HPV-45 0.50.

Regarding family planning method, it was found that using hormonal contraceptives increased normalised viral load by values close to unity on the logarithmic scale for HPV-16, -18 and -31 types, the maximum value being that reached by HPV-18 (coef. 1.06: 0.22 to 1.90 95%CI; p= 0.013).

The use of any type of family planning method increased normalised viral load in the HPV-16 infection, this being 0.57 for the use of methods different to the hormonal one.

Presence and absence values were included regarding abortion, finding that HPV-45-infectad women having a background of abortion represented increased viral load by 0.39 logarithmic units.

A clinical factor evaluated in the women in the study was the presence of other STDs. This factor's effect on the cohort was observed in the load for women having infection

different to HIV and the presence of HPV-45, since concomitance with other infections became reduced by almost 1 logarithmic unit.

Regarding multiple infection modulating viral load, it was found that the increase in each number of types of HPV for women having HPV-45 infection increased their load by 0.47 logarithmic units.

Age on beginning sexual relations and the number of children had no effect regarding the modulation of normalised viral load for this cohort.

Discussion

This work has consolidated cervical sample viral load dynamics in a population of Colombian women who were followed-up every 6 months over a period of at least 2 years.

The mean for the female population regarding this follow-up consisted of middle-aged women, permitting this work to broaden the currently available information; this is relevant regarding the recent introduction of screening for DNA from HPV.

These infection dynamics have shown the changes in the number of viral types per cell, concerning 4 to 5 samples taken over a 2-year period.

This work has shown that absolute loads for HPV-18 had constant means in the first three follow-ups. A recently published study by our group determined this type's major viral persistence in the same cohort [10]. The foregoing agreed with findings established in a cohort of adult women where persistent infections' viral loads were similar regarding follow-up time, regardless of persistence duration [12].

It was not easy to establish parameters affecting the reduction of normalised load for HPV-16, -18 and -45-infected women from cities other than Bogotá since aspects regarding customs/habits were not measured in the study and may have influenced infection by these types. The differences between Girardot and Bogotá have been identified previously regarding HPV prevalence, type-specific distribution, multiple infection and the time taken for determining viral clearance [13]. In spite of the above, data for HPV-18 was only correlated with persistence.

It has been described that viral loads have increased rapidly in women having some degree of lesion in populations studied around the world, leading to the association of increased viral load with greater risk of developing lesions [14]. It is worth stating that this has been demonstrated in patients having pre-neoplastic lesions; however, types like HPV-16 had the worst colposcopy prognoses regarding low load values in our cohort [10].

Having had a larger number of sexual partners was another factor involved in reducing viral load involving the two most representative types of HPV from the A7 species (HPV-18 and -45). Such data could lead to inferring that an increased number of sexual partners favoured regulation of viral load in such a way that it had no effect on recognition by the immune system and this form in viral clearance.

Competition between HPV genotypes has been suggested; reduction of pre-existing types' (HPV-16 and HPV-18) viral load has been reported when infection by a type from the same species has been acquired [8]. Even though different viral types may coexist in the same cell population or in the same cell, there are interactions between them [15]. Viral types could compete in the cervical epithelium for factors which are necessary for replication, transcription, translation and/or persistence [8]. It has been

described that two types competing for the pool of cell factors could affect the replication of both genomes, or just one viral type due to competition, being shown by the difference in viral load [15]. Having had more sexual partners was an indicator of possible new acquisition of viral types, thereby explaining the reduction of these two types from the same species.

Nevertheless, our study also found an increase in HPV-45 viral load modulated by the increase in viral types from multiple infections. Increased viral load in the presence of other types would probably indicate that this type's replication was favoured due to the aforementioned competition since more viral types (4 of the types evaluated belonged to the A9 species) from another species would modulate such characteristic.

Analysing the data arising from this cohort would suggest that medium to low viral loads tend not to be detectable by the immune system; it could thus be thought that women suffering infection having these characteristics would have greater persistence as time elapses, which was shown by HPV-18 which had the greatest persistence in the cohort.

It is not uncommon to observe reduced HPV-45 load in the presence of other STDS, as it has been described that agents such as *Chalmydia trachomatis* and herpes simplex virus type 2 (HSV-2) have been associated with the risk of developing CC [16]. Due to the foregoing, and bearing in mind that persistence in this cohort was related to low viral loads, then there could have been "equilibrium" in the women infected with additional STD thereby and that such concomitant infections go unnoticed by the immune system.

It has also been reported that the presence of HPV-16 and HPV-18 types in the cervix tends to reduce the number of Langerhans cells, these being the active antigen-

presenting cells in the layers of the cervical mucosal epithelia [17]. The foregoing could justify immune system invisibility and the lower cost of replication for the types infecting the tissue.

Regarding increased viral load, hormonal contraceptive use was the variable having the greatest number of related types, thereby increasing HPV-16, -18 and -31 viral load. Something similar was found in a cohort of young women in which HPV-18 had markedly greater loads in women who used a hormonal planning method [5]. It has been shown that the proteoglycans in this type of contraceptive activate viral replication promoters, meaning that increased load due to this effect is understandable [18,19].

Interestingly, being white had an effect on considerably increasing HPV-18 and -58 viral load. It has been indicated that some viral types could have a preference for infecting determined ethnicities as there are behavioural patterns in the different ethnic groups supporting such preference [20]. It has been described that the molecular variants for types like HPV-16 and -18 are more likely to affect the populations from which they come, for example, European variants would tend to prefer white women [21]. Unfortunately, this study did not determine the variants involved and this could form the basis for future research regarding this population.

Having had an abortion was related to increased viral load for HPV-45; however, this study did not distinguish whether the abortions had been spontaneous or induced. The pertinent literature has reported the possible role of HPV in spontaneous abortion due to trophoblast alteration which would alter pregnancy [22]. A direct relationship between having had an abortion and viral load incidence has not been reported to date, to the best of our knowledge. Nevertheless, greater association of women having a background of abortion and multiple infection has been reported possibly explaining greater lesions in

the cervical mucosa or a high risk sexual life (frequent sexual activity without protection) [23]. The foregoing could explain this relationship found in our analysis, given that HPV-45 was the only type which increased its load in the presence of other coinfectant types.

Age influenced increased HPV-31 load. Some reports have suggested reduced HR-HPV prevalence as increased load would be involved in processes different to that promoting lesion development as age increased and within the framework of our observations. HPV-31 has a high prevalence in Italy, following that for HPV-16. The present study determined high viral loads overall which were associated with lesion development; however, evidence of high viral load for this type could form the basis for prognosis regarding lower oncogenic risk for developing high-grade squamous intraepithelial lesions (H-SIL) [24].

Previous studies have highlighted the need for understanding the dynamics for the different types of HR-HPV; the present work has shown that different factors affect different viral types' loads. This type of study is necessary in populations having many CC-related morbidity-mortality cases where aspects are being identified which should lead to improvements in primary and secondary cancer prevention programmes.

Conclusions

Few cohort studies have recorded HR-HPV viral loads for types different to HPV-16 and/or -18. This has been the first study in Colombia which has used RT-PCR for determining viral load.

Some risk factors were involved in modulating the viral load of 6 types of HR-HPV and interfered in different ways for each type evaluated here. Each time these factors

modulated multiple viral types, the load was regulated in the same way for each viral type, all either increasing or decreasing their load.

It was found that certain factors influenced the reduction of viral load, such as not living in Bogotá for HPV-16, -18 and -45, the greater number of sexual partners for HPV-18 and -45 and concomitant sexually-transmitted diseases other than HIV for HPV-45. Factors related to increased normalised viral load involved using any type of family planning method for HPV-16 infected women and using hormonal methods regarding HPV-18 and -31. HPV-18 or -58 load became considerably increased in white women regarding mestizo population. Advanced age was associated with greater viral load in HPV-31 infected women. Multiple infections and having a background of abortions increased the viral load for HPV-45 infected women.

Acknowledgements. We would like to thank the people who collaborated in obtaining the patients who participated in this study, Darwin Andres Moreno and Claudia Marina Muñoz for their assistance in the methodological part of the study.

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Figure legend

Figure 1. HPV median viral loads. Absolute viral load dynamics according to visit.

Tables

Table 1. Population description.

Sociodemographic data		HPV types % (n=) [IC 95%]					
Variable	**Category**	**16**	**18**	**31**	**33**	**45**	**58**
City	Bogotá	26% [a](40) [19 - 33]	25% (29) [18-34]	48% (39) [37-59]	36% (5) [13-65]	20% (18) [12-29]	41% (41) [31-52]
	Chaparral	3% (4) [0.7 -6.4]	3% (3) [0.5-7.4]	1% (1) [0.03-7]	-	3% (3) [0.6-9]	3% (3) [1-8]
	Girardot	71% (112) [64-79]	72% (82) [63-80]	51% (41) [39-62]	64% (9) [35-87]	77% (70) [67-85]	56% (56) [46-66]
Age	<35	22% (35) [16- 30]	27% (31) [19-36]	25% (20) [16-36]	36% (5) [13-65]	88% (82) [80-94]	24% (24) [16-34]
	35-44	35% (54) [27- 43]	30% (34) [22-39]	25% (20) [16-36]	14% (2) [2-43]	1% (1) [0.02-6]	30% (30) [21-40]
	45-55	30% (47) [23- 38]	33% (38) [25-43]	39% (32) [29-51]	43% (25) [17-71]	3% (3) [1-9]	39% (39) [29-50]
	>55	13% (20) [8-19]	10% (11) [5-16]	11% (9) [5-20]	7% (4) [0.1-34]	8% (7) [3-15]	7% (7) [3-14]
Ethnic group	Mestizo	96% (150) [92-98]	97% (111) [93-99]	99% (80) [93-99]	100% (14)	96% (89) [89-99]	95% (95) [89-98]
	White	2% (39 [0.3- 5.5]	2% (2) [0.2-6]	-	-	2% (2) [0.2-8]	2% (2) [0.02-7]
	Indigenous	0.6% (1) [0.1- 3.5]	-	1% (1) [0.03-7]	-	1% (1) [0.02-6]	2% (2) [0.02-7]
	Afro-descendant	1.4% (2) [0.1- 4.5]	1% (1) [0.02-5]	-	-	1% (1) [0.02-6]	1% (1) [0.02-5]
Age on first sexual relation	<=18	58% (90) [50-66]	50% (57) [40-60]	58% (47) [47-69]	50% (7) [23-77]	65% (60) [54-74]	58% (58) [48-68]
	>18	42% (66) [34-50]	50% (57) [40-60]	42% (34) [31-53]	50% (7) [23-77]	35% (33) [26-46]	42% (42) [32-52]
Sexual partners	1	47% (73) [39-55]	47% (53) [37-56]	49% (40) [38-60]	43% (6) [18- 71]	45% (42) [35-56]	42% (42) [32-52]
	2	37% (58) [30-45]	33% (37) [24-42]	31% (25) [21-42]	50% (7) [23-77]	35% (33) [26-46]	34% (34) (25-44]
	>2	16% (25) [11-23]	20% (22) [11-26]	20% (16) [12-30]	7% (1) [0.01- 34]	20% (18) [12-29]	24% (24) [16-34]
Family	No Method	43% (68) [36%-52%]	43% (45) [30-49]	42% (34) [31-53]	21% (3) [4-50]	39% (36) [29-49]	48% (48) [38-58]

planning method	Hormonal	10% (15) [5.4-15]	12% (13) [6.2-19]	11% (9) [5-20]	43% (6) [18-71]	16% (15) [9-25]	10% (10) [5-17]
	Other	47% (73) [39-55]	45% (48) [33-52]	47% (38) [36-58]	36% (5) [13-65]	45% (42) [35-56]	42% (42) [32-52]
Children	None	4% (6) [1.4 - 8.1]	4% (5) [1.4-10]	5% (4) [1-12]	7% (1) [0.1-34]	5% (5) [1-12]	3% (3) [0.6-9]
	1	23% (36) [17-30]	18% (20) [11-26]	52% (42) [40-63]	29% (4) [8-58]	14% (13) [7-23]	24% (24) [16-34]
	>1	73% (114) [65-80]	78% (89) [69-85]	43% (35) [32-55]	64% (9) [35-87]	81% (75) [71-88]	73% (73) [63-81]
Abortion	No	62% (92) [53-69]	65% (70) [55-74]	65% (51) [54-76]	71% (10) [42-92]	66% (57) [55-76]	61% (61) [51-71]
	Yes	38% (57) [30-47]	35% (38) [26-45]	35% (27) [24-46]	29% (4) [8-58]	34% (29) [24-45]	39% (39) [29-49]
STD[b]	None	83% (129) [76-88]	79% (87) [70-86]	79% (64) [69-87]	100% (14)	81% (71) [71-88]	84% (84) [75-91]
	HIV[c]	1% (1) [0.01-3.5]	1% (1) [0.02-5]	1% (1) [0.03-7]	-	1% (1) [0.02-6]	1% (1) [0.02-5]
	Other	16% (26) [11-23]	20% (22) [13-29]	20% (16) [12-30]	-	18% (16) [11-27]	15% (15) [9-24]
Cytology	Negative	90% (140) [84-94]	94% (107) [88-97]	89% (72) [80-95]	93% (13) [66-99]	94% (87) [86-98]	90% (90) [82-95]
	ASCUS[d]	4% (7) [2-9]	3% (4) [0.9-8.7]	6% (5) [2-13]	7% (1) [0.1-34]	3% (3) [0.7-9]	7% (7) [3-14]
	LSIL[e]	6% (9) [3-10]	3% (3) [0.5-7.4]	5% (4) [1-12]	-	3% (3) [0.7-9]	3% (3) [0.6-9]
	HSIL[f]	-	-	-	-	-	-
Colposcopy	Negative	78% (119) [70-84]	78% (78) [68-86]	81% (66) [71-89]	86% (12) [57-98]	76% (7) [66-84]	96% (96) [90-98]
	LSIL[e]	1% (1) [0.01-3.5]	21% (21) [13-30]	15% (12) [8-24]	14% (2) [2-43]	3% (3) [0.6-9]	4% (4) [1-10]
	HSIL[f]	21% (33) [15-29]	1% (1) [0.02-5.4]	-	-	21% (19) [13-30]	-
Multiple Infection	Yes	90% (140) [84-94]	89% (102) [82-94]	90% (73) [81-96]	100% (14)	3% (3) [0.6-9]	87% (87) [78-92]
	No	10% (16) [6-16]	11% (12) [6-18]	10% (8) [4-18]	-	97% (90) [91-99]	13% (13) [7-21]
Types	1	11% (17) [6.4-17]	10% (12) [6-18]	10% (8) [4-18]	-	4% (4) [1-10]	13% (13) [7-21]
	2	22% (35) [16-30]	17% (19) [10-25]	18% (15) [11-29]	14% (2) [2-43]	12% (11) [6-20]	29% (29) [20-39]
	3	39% (60) [31-47]	38% (43) [29-47]	22% (18) [14-33]	7% (1) [0.1-34]	56% (52) [45-66]	21% (21) [13-30]
	4	18% (28) [12-25]	24% (28) [17-33]	30% (24) [20-40]	14% (2) [2-43]	11% (10) [5-19]	23% (23) [15-32]
	5	7% (11) [3.5-12]	7% (8) [3-13]	14% (11) [7-23]	29% (4) [8-58]	12% (11) [1-20]	9% (9) [4-16]
	6	3% (5) [1-7.3]	4% (4) [1-9]	6% (5) [2-14]	36% (5) [13-65]	5% (5) [2-12]	5% (5) [2-11]

Sociodemographic and clinical description for each HPV type.

[a] Percentages correspond to infection events with each HPV type, calculated over the total of 219 infections, given that some women were coinfected.

[b] STD: Sexually-transmitted diseases

[c] HIV: human immunodeficiency virus

[d] ASCUS: Atypical squamous cells of undetermined significance

[e] L-SIL: Low-grade squamous intraepithelial lesions

[f] H-SIL: High-grade squamous intraepithelial lesions

Table 2. GEE results.

Sociodemographic data		**Model for each HPV type**														
Variable	*Category*	**HPV-16**			**HPV-18**			**HPV-31**			**HPV-45**			**HPV-58**		
		Coefficient	**p**	**95%CI**	**Coefficient**	**p**	**95%CI**	**Coefficient**	**p**	**95%CI**	**Coefficient**	**p**	**95%CI**	**Coefficient**	**p**	**95%CI**
City	*Bogotá*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Other	**(-) 0.52**	**0.019**	**[-0.95 to 0.08]**	**(-) 0.91**	**0.001**	**[-1.44 to - 0.38]**	(-)0.004	0.988	[-0.59 to 0.58]	**(-) 0.63**	**0.007**	**[- 1.09 to -0.17]**	(-) 0.28	0.594	[-1.31 to 0.75]
Age		0.01	0.54	[-0.01 to 0.3]	0.02	0.092	[- 0.00 to 0.05]	**0.05**	**0.001**	**[0.02 to 0.08]**	0.007	0.187	[-0.003 to 0.018]	0.008	0.785	[-0.05 to 0.06]
Ethnic group	*Mestizo*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	White	0.44	0.55	[-1.01 to 1.89]	**2.16**	**0.039**	**[0.11 to 4.23]**	N/A	N/A	N/A	(-) 0.24	0.721	[-1.60 to 1.11]	**4.82**	**0.001**	**[2.06 to 7.57]**
	Indian	(-) 0.60	0.47	[-2.23 to 1.02]	N/A	N/A	N/A	1.46	0.302	[-1.31 to 4.24]	1.24	0.146	[-0.43 to 2.91]	-0.295	0.872	[-3.88 to 3.29]
	Afro-descendant	1.83	0.11	[-0.41 to 4.07]	1.27	0.272	[-1.00 to 3.56]	N/A	N/A	N/A	0 .04	0.954	[-1.32 to 1.40]	(-) 0.25	0.901	[-4.09 to 3.61]
Age on first sexual relation	*<=18*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	>18	0.01	0.95	[-.39 to 0.41]	0.16	0.522	[-0.33 to 0.65]	(-) 0.414	0.144	[-0.97 to 0.142]	-0.11	0.529	[-0.48 to 0.25]	(-) 0.15	0.770	[-1.14 to 0.84]
Sexual partners	*None*	N/A	N/A	N/A	0.60	0.832	[-4.95 to 6.15]	N/A	N/A	N/A	(-) 0.70	0.743	[-4.9 to 3.5]	0.713	0.806	[-4.98 to 6.41]
	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	2	0.01	0.97	[-0.42 to 0.43]	0.14	0.601	[-0.39 to 0.68]	0.144	0.654	[-0.48 to 0.77]	**(-) 0.50**	**0.018**	**[-0.92 to -0.08]**	0.83	0.117	[-0.21 to 1.87]
	More than 2	0.14	0.59	[-0.38 to 0.66]	**-0.69**	**0.034**	**[-1.33 to 0.05]**	(-) 0.700	0.084	[-1.49 to 0.09]	(-) 0.13	0.616	[-0.65 to 0.38]	0.62	0.327	[-0.62 to 1.87]
Family planning method	*No method*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Hormonal	**0.83**	**0.01**	**[0.19 to 1.47]**	**1.06**	**0.013**	**[0.22 to 1.90]**	**0.923**	**0.046**	**[0.02 to 1.83]**	(-) 0.10	0.743	[-0.67 to 0.48]	0.96	0.214	[-0.55 to 2.48]
	Other	**0.57**	**0.01**	**[0.13 to 1.0]**	0.58	0.060	[-0.024 to 1.19]	0.40	0.262	[- 0.30 to 1.10]	(-) 0.20	0.341	[-0.62 to 0.22]	(-) 0.54	0.310	[-1.61 to 0.51]
Children	*None*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1	0.16	0.74	[-0.81 to 1.14]	0.75	0.261	[-0.55 to 2.05]	(-) 1.25	0.125	[- 2.85 to 0.35]	0.53	0.243	[-0.36 to 1.43]	(-) 1.78	0.254	[-4.84 to 1.28]
	>1	(-) 0.12	0.81	[-1.08 to 0.84]	(-) 0.07	0.915	[-1.34 to 1.19]	(-)1.69	0.051	[-3.39 to 0.004]	0.67	0.092	[- 0.11 to 1.45]	(-) 0.44	0.763	[-3.31 to 2.43]
Abortion	*No*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Yes	(-) 0.04	0.84	[-0.43 to 0.34]	(-) 0.18	0.446	[-0.67 to 0.29]	0.12	0.666	[-0.45 to 0.71]	**0.39**	**0.037**	**[0.02 to 0.76]**	(-) 0.72	0.136	[-1.66 to 0.23]
STD[a]	*None*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	HIV[b]	0.86	0.39	[-1.13 to 2.86]	1.80	0.148	[-0.64 to 4.24]	1.43	0.203	[- 0.77 to 3.63]	0.24	0.772	[-1.41 to 1.89]	0.65	0.828	[-5.23 to 6.54]
	Other	(-) 0.60	0.053	[-1.21 to 0.01]	(-) 0.58	0.165	[-1.40 to 0.24	(-) 0.01	0.988	[-0.77 to 0.76]	**(-) 0.90**	**0.013**	**[-1.60 to -0.19]**	(-) 0.24	0.757	[-1.78 to 1.29]
Types		0.021	0.767	[- 0.12 to 0.17]	0.08	0.477	[- 0.13 to 0.28]	0.144	0.165	[-0.06 to 0.35]	**0.47**	**0.000**	**[0.32 to 0.63]**	(-) 0.04	0.857	[-0.42 to 0.35]

Normalised viral load for each HPV type.

[a] STD: Sexually-transmitted diseases

[b] HIV: human immunodeficiency virus

CONCLUSIONES, RECOMENDACIONES Y PERSPECTIVAS GENERALES

Conclusiones

La inclusión de una población culturalmente y geográficamente diversa, en comparación con previos estudios llevados a cabo en Colombia, permitió la detección de asociaciones importantes entre la etnia y la frecuencia de infección por VPH.

Con el ánimo de cumplir con el primer objetivo específico planteado se describieron las características socio-demográficas y clínicas de las mujeres que participan en este estudio. Se trabajó con una población vulnerable de nuestro país, en su mayoría mujeres entre los 17-69 años, mestizas, con una relación en unión libre con su pareja y principalmente mujeres cuyos ingresos no superan el salario mínimo. En esta población de estudio no se incluyeron mujeres con cáncer y muy pocas presentaron lesiones cervicales de alto grado.

Se determinó que poblaciones como Girardot y Leticia presentan las mayores frecuencias de infecciones múltiples y que la etnia indígena está más susceptible a infecciones por más de un tipo viral. Metodologícamente se pudo comprobar que el set de cebadores MY09/11 permite la identificación de las infecciones múltiples eficientemente.

La prevalencia de VPH en el grupo de mujeres participantes en el estudio, fue del 42% y las prevalencias tipo-específicas de los VPH-AR identificados en esta población fueron: VPH-16= 36.1%, VPH-18=15.1%, VPH-31=18.6%, VPH-33=12.3%, VPH-45=10.7%, VPH-58=7%.

En todo el estudio el tipo viral más frecuente fue VPH-16, seguido de VPH-31 en aquellas poblaciones con frecuencias altas de infecciones múltiples. El análisis molecular permitió identificar una alta prevalencia de VPH-31, lo cual no había sido

reportado previamente para Colombia, adicionalmente este tipo viral estuvo asociado a coinfecciones con VPH-33.

El uso de más de un set de identificación viral aumenta la sensibilidad y brinda mayor robustez en los estudios epidemiológicos.

La prevalencia de VPH por la técnica de Captura Hibrida (HC2) fue 36.5%, mientras que por PCR fue 55.2%. Donde la mayor concordancia de HC2 se presentó en la identificación de VPH con el set de primers genéricos pU1M/2R, set que mostró el mejor rendimiento diagnóstico.

La mayor persistencia de los tipos evaluados en este trabajo, se encontró para VPH-18 y -31 y los valores de depuración más rápidos los presentaron las mujeres infectadas con VPH-16 y -33. Todos los tipos virales presentaron eventos de reinfección en el 40% de los casos en promedio, siendo el más bajo para VPH-18 y nulo en el caso de VPH-58.

Con respecto a los factores sociodemográficos relacionados con la depuración, se encontró que aquellas mujeres infectadas con los tipos VPH-16 y -58 que no residían en Bogotá, tuvieron eventos de eliminación viral más rápidos que aquellas residentes en la capital. Adicionalmente se pudo relacionar valores bajos de carga viral normalizada, para VPH-31 con persistencia del virus y peor pronóstico en el hallazgo colposcópico para VPH-16 en mujeres con bajos valores de carga absoluta.

La modulación de la carga viral de los seis tipos de VPH-AR evaluados se dio diferencialmente según el tipo presente en la infección.

En la cuantificación del ADN de VPH existe una baja relación de las infecciones múltiples con la modulación de la carga viral para 5 de los 6 VPH-AR evaluados. Se logró determinar que las mujeres infectadas por VPH-45 y que presentaban infecciones múltiples pudieron experimentar un aumento en la carga viral para este tipo a medida que se agregaban más tipos virales a la infección.

Recomendaciones

Para tener una identificación robusta de los datos de depuración del VPH-AR sería importante el diseño de una cohorte con tiempos más cortos entre los seguimientos, ya que la presencia del virus podría evaluarse más veces en el tiempo.

Con el objeto de correlacionar datos de carga viral con desenlace de enfermedad, habría que establecer una cohorte más larga, dado que determinar anormalidades cervicales, según la historia natural de la infección requiere más de 5 años. Adicionalmente las dinámicas de las infecciones, en un tiempo mayor serían mejor caracterizadas, ya que hubo tipos con bajas prevalencias como el HPV-33, que fueron subdeterminados en el tiempo que duró esta cohorte.

La determinación de variantes moleculares para VPH-16 y -18 permite identificar aquellas variantes involucradas en la persistencia en nuestra población, aquellas que se depuran fácilmente y las prevalencias de éstas en población heterogénea como la nuestra.

Realizar estudios de cohorte en población vulnerable y alcanzar mayores tamaños muestrales en mujeres indígenas y afrodescendientes, con el fin de enfatizar los rasgos determinantes de las etnias en la población Colombiana y su asociación con las infecciones por VPH.

Perspectivas Generales

Un factor interesante que no fue posible tener en cuenta en este estudio fue el análisis de muestras aportadas por las parejas sexuales de las mujeres incluidas en los estudios, lo cual es importante para comprender claramente la dinámica de las cargas en el contexto práctico y aplicado de la infección.

Una perspectiva que se tiene es la posibilidad de realizar análisis genéticos (ej: identificación de HLA clase I y II) de las poblaciones étnicas colombianas, con la finalidad de aclarar los aspectos relacionados con etnia e infección por VPH.

La importancia que en los últimos años ha tenido el descubrimiento de VPH en diferentes tipos de cánceres inspira a trabajar con otros tejidos (muestras de Ca de colon, ano) para comenzar a delimitar las frecuencias y las causas de diferentes cánceres en poblaciones socio-demográfica y culturalmente diferentes como la colombiana.

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