



**UNIVERSIDAD DEL ROSARIO**



**FIDIC**

**ASOCIACIÓN DEL VIRUS DEL PAPILOMA HUMANO CON LA PRESENCIA  
DE DOS INFECCIONES DE TRANSMISIÓN SEXUAL EN MUJERES  
COLOMBIANAS**

**SANDRA MILENA CAMARGO PINZÓN B.Sc., M.Sc.**

“Documento de tesis presentado como requisito para optar al título de Doctor en Ciencias  
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**UNIVERSIDAD DEL ROSARIO  
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BOGOTÁ D.C.  
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**Estudiante**

**Sandra Milena Camargo Pinzón B.Sc., M.Sc.**

Bacterióloga, Universidad Colegio Mayor de Cundinamarca  
Magíster en Genética Humana, Universidad del Rosario  
Investigadora principal  
Fundación Instituto de Inmunología de Colombia (FIDIC)

**Director**

**Manuel Alfonso Patarroyo Gutiérrez M.D., Dr.Sc.**

Profesor Titular, Escuela de Medicina y Ciencias de la Salud  
Universidad del Rosario  
Jefe del Departamento de Biología Molecular e Inmunología  
Fundación Instituto de Inmunología de Colombia (FIDIC)

**Co-Director**

**Sara Cecilia Soto de León B.Sc., Dr.Sc.**

Fundación Instituto de Inmunología de Colombia (FIDIC)

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## DEDICATORIA

*A mis padres y a mi hermana*

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## LISTA DE ABREVIATURAS

<b>Abreviatura</b>	<b>Término</b>
<b>ADC</b>	Adenocarcinoma
<b>ADN</b>	Ácido Desoxirribonucleico
<b>ADN</b>	Ácido Desoxirribonucleico
<b>ARN</b>	Ácido Ribonucleico
<b>ART</b>	de la sigla en inglés: Antiretroviral Therapy
<b>ASC-US</b>	Células Escamosas Atípicas de Significado Indeterminado
<b>CC</b>	Cáncer de Cérvix
<b>CCE</b>	Carcinoma de Células Escamosas
<b>CE</b>	Cuerpo Elemental
<b>CR</b>	Cuerpo Reticulado
<b>Ct</b>	<i>Chlamydia trachomatis</i>
<b>CV</b>	Carga Viral
<b>E</b>	de la sigla en inglés: Early
<b>ICTV</b>	de la sigla en inglés: International Committee on Taxonomy of Viruses
<b>ITS</b>	Infecciones de Transmisión Sexual
<b>L</b>	de la sigla en inglés: Late
<b>LCR</b>	Long Control Region
<b>LEI</b>	Lesión Intraepitelial Escamosa
<b>LEIAG</b>	Lesión Intraepitelial Escamosa de Alto Grado
<b>LEIBG</b>	Lesión Intraepitelial Escamosa de Bajo Grado
<b>LGV</b>	Linfogranuloma Venéreo
<b>MLST</b>	de la sigla en inglés: Multilocus sequence typing
<b>MLVA</b>	Multiple loci VNTR analysis
<b>NIC</b>	Neoplasia Intraepitelial Cervical
<b>PCR</b>	de la sigla en inglés: Polymerase Chain Reaction
<b>pRB</b>	Proteína Retinoblastoma
<b>qPCR</b>	de la sigla en inglés: Quantitative Real Time Polymerase Chain Reaction
<b>RFLP</b>	de la sigla en inglés Restriction Fragment Length Polymorphism
<b>VIH</b>	Virus de la Inmunodeficiencia Humana
<b>VNTR</b>	De la sigla en inglés: Variable Number of Tandem Repeats
<b>VPH</b>	Virus del Papiloma Humano
<b>VPH-AR</b>	Virus del Papiloma Humano de Alto Riesgo
<b>VPH-BR</b>	Virus del Papiloma Humano de Bajo Riesgo
<b>WHO</b>	de la sigla en inglés: World Health Organization

## RESUMEN

Las Infecciones de Transmisión Sexual (ITS) son una causa importante de morbilidad, generando gran impacto en la salud pública por las secuelas a las que conllevan. La importancia a nivel clínico de éstas radica en que su persistencia ha sido asociada con daño progresivo y continuo de la arquitectura cervical, que conduce al desarrollo de lesiones que pueden progresar a neoplasia cervical. El Virus del Papiloma Humano, es la ITS viral de mayor distribución, siendo ampliamente demostrada su asociación con el desarrollo de Cáncer Cervical (CC). Se ha descrito que factores adicionales, como el comportamiento sexual del hospedero, factores intrínsecos del VPH y la coexistencia de otras ITS, podrían estar desempeñando un rol relevante en el desarrollo de la enfermedad cervical.

En este estudio, se analizó la coexistencia entre VPH y dos ITS de amplia distribución, *Chlamydia trachomatis* y el Virus de Inmunodeficiencia Humana (VIH). Este proyecto de investigación fue diseñado en dos componentes; el primero, de tipo longitudinal, buscó determinar la incidencia de infección por *C. trachomatis* y su relación con la presencia y carga viral de 6 tipos de VPH de alto riesgo (VPH-AR-16, -18, -31, -33, -45 y -58), en mujeres provenientes de tres ciudades de Colombia. Los resultados mostraron que VPH-18 y -31 fueron los tipos más persistentes, mientras que VPH-16 y -33 se eliminaron en menor tiempo. En cuanto a la carga viral (CV), se encontró que una baja CV de VPH-16 y -31 se asoció con una mayor frecuencia de Lesión Intraepitelial Escamosa (LEI).

En cuanto a *C. trachomatis*, una cuarta parte de las mujeres del estudio iniciaron con infección bacteriana, aumentando su frecuencia de aparición a lo largo de los seguimientos; mujeres con factores como la presencia de múltiples tipos de VPH, mayor número de compañeros sexuales y antecedentes de aborto, presentaron un mayor riesgo de adquirir esta infección bacteriana. En cuanto a efecto de la CV en el curso clínico de *C. trachomatis*, los resultados mostraron que las variaciones en el número de copias virales influyen en el desenlace de las infecciones bacterianas; adicionalmente, estos efectos fueron dependientes del tipo de VPH infectante.

El segundo componente, corresponde a un estudio de corte transversal, con el cual se buscó establecer la frecuencia de la infección por VPH en un grupo de mujeres colombianas que conviven con el VIH. Con este estudio, se encontraron elevadas frecuencias de infección y coinfección (definida como la detección simultánea de múltiples tipos de VPH) en mujeres que conviven con VIH; factores como el recuento de células CD4 y la carga viral de VIH, se asociaron positivamente a la infección y coinfección de VPH, mientras que el tratamiento antiretroviral se asoció sólo con las infecciones por VPH.

Los resultados de este estudio indican que la infección y coinfección de VPH, se ven influenciadas por un microambiente cervical, en el que factores del hospedero (como el comportamiento sexual) y la presencia de otros patógenos que comparten la misma ruta de transmisión, coexisten e interactúan favoreciendo la permanencia, replicación e integración del VPH en el hospedero, y trayendo como consecuencia la aparición de anormalidad a nivel cervical.

La relación causal entre infecciones por VPH y el desarrollo CC ha sido ampliamente demostrada; características propias del VPH influyen en el curso clínico de dichas infecciones. Sin embargo, la infección con VPH por sí sola no es suficiente para que se desarrolle esta neoplasia, por lo que otras características como la coexistencia con otras ITS, constituyen un factor clave. La comprensión del papel que desempeñan las ITS distintas a VPH en el desarrollo de CC resulta, por ende, relevante y plantea nuevos retos en el abordaje e implementación de estrategias acertadas que mejoren y prolonguen la calidad de vida en la población femenina.

## ESTADO DEL CONOCIMIENTO

### *Infecciones de Trasmisión Sexual*

Las infecciones de transmisión sexual (ITS) son un importante problema de salud pública, principalmente en países en vías de desarrollo, donde afectan en mayor proporción al género femenino (1). A diferencia de otras enfermedades transmisibles, donde las estrategias de promoción y prevención han conducido a la reducción de las tasas de morbi-mortalidad, para el caso de las ITS no se ha logrado disminuir su impacto a nivel mundial; como consecuencia, afecciones urogenitales agudas como cervicitis, uretritis y vaginitis, conducen a complicaciones graves a corto y largo plazo, como la enfermedad pélvica inflamatoria, embarazo ectópico y la infertilidad, entre otras (2).

Diferentes microorganismos han sido descritos, cuya ruta de transmisión es por vía sexual; los más comunes son el Virus del Papiloma Humano (VPH), *Chlamydia trachomatis*, Virus de Inmunodeficiencia Humana (VIH), *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, *Herpes simplex* y *Treponema pallidum* (3). En relación a su impacto, se han reportado cerca de 376 millones de casos de nuevas infecciones a nivel mundial para cuatro de las ITS consideradas curables (*C. trachomatis*, *T. vaginalis*, *N. gonorrhoeae* y *T. pallidum*). 1,7 millones de personas conviven con VIH y aproximadamente 291 millones de mujeres con infecciones por VPH (4, 5).

Cabe destacar, que muchas de estas infecciones no son diagnosticadas y permanecen asintomáticas, por lo que no son tratadas a tiempo y son fácilmente transmitidas después del contacto sexual (6). La rápida propagación de estas ITS junto al uso excesivo de antibióticos, ha conducido a que en bacterias como *C. trachomatis* y protozoos como *T. vaginalis*, hayan emergido cepas multirresistentes, lo que genera un problema adicional en su tratamiento (7).

Grandes avances técnicos como la inclusión de pruebas moleculares para el diagnóstico, han permitido una mejora en los programas de detección temprana de las ITS; sin embargo, dada la carga de morbilidad y mortalidad de estas infecciones, aún son consideradas un problema a nivel de salud pública, siendo el comportamiento sexual (promiscuidad, falta de protección, entre otros), el factor de riesgo asociado de mayor

importancia para su adquisición y rápida dispersión en la población (8-10). Las ITS siguen siendo las enfermedades infecciosas más diseminadas y peligrosas (11-13), las cuales tienen un gran impacto en la salud sexual y neonatal, siendo algunas de ellas asociadas con afecciones graves como el cáncer cervical (14, 15).

Cerca del 18% de los cánceres se han asociado con la presencia de agentes infecciosos, entre ellos, el *Helicobacter pylori*, el virus de Epstein-Barr, el virus de la hepatitis y el VPH, no obstante, buena parte de estas infecciones cursan sin cambios clínicos que deriven en sintomatología asociada a su presencia o neoplasias, por lo que sus hospederos favorecen la continuidad de los ciclos de transmisión (13). En este contexto, factores adicionales como otras infecciones concomitantes, pueden contribuir en la patogénesis del desarrollo de neoplasias, generando un efecto sinérgico que conlleva a la inducción de hipertrofia, daño de la integridad celular, alteraciones en la reorganización de la actina y transformación del tejido cervical por la acumulación de factores involucrados en los procesos inflamatorios (16-18).

Estudios han mostrado que algunos tipos de cáncer tienen un origen infeccioso, por lo que los microorganismos son uno de los factores clave que contribuyen en el desarrollo de anormalidades a nivel celular (11, 12). Infecciones por estos microorganismos causan inestabilidad genómica en el hospedero, daños en la respuesta inmune, entre otras alteraciones (19). Adicionalmente, éstos pueden integrarse aleatoriamente en distintas regiones del genoma del hospedero, trayendo como consecuencia la metilación del ADN, modificación de histonas, alteración del nivel de miARN, entre otros daños a nivel genético (20-22).

### ***Cáncer de Cérvix***

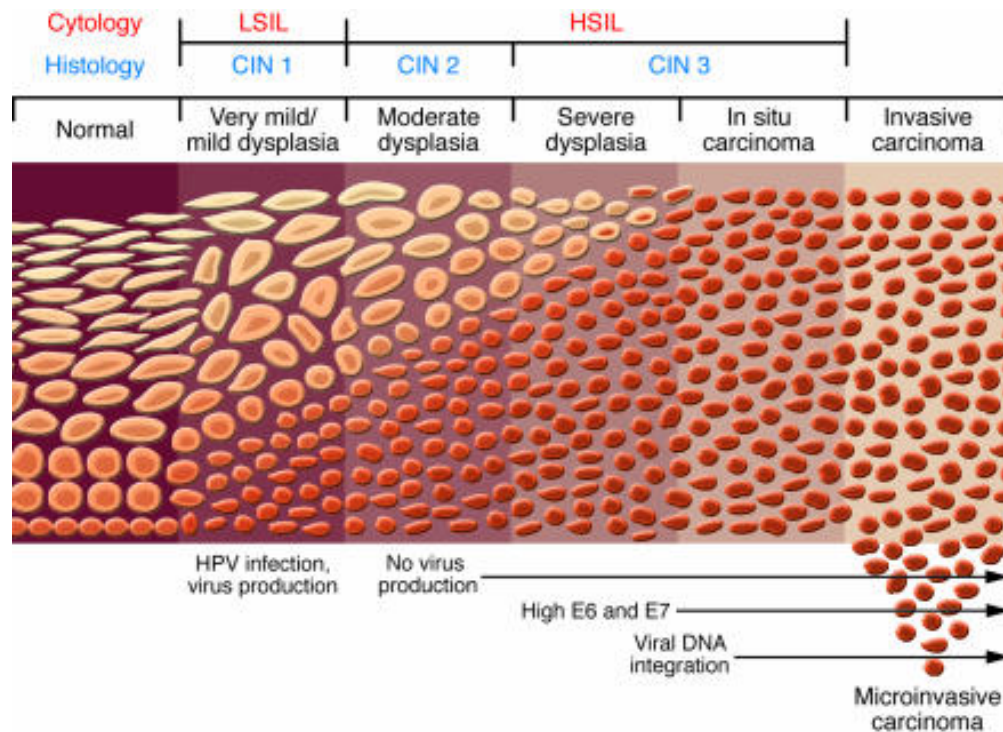
El Cáncer de Cérvix (CC) es considerado un grave problema de salud pública. Entre los distintos cánceres, ocupa el séptimo lugar en frecuencia a nivel global, mientras que en mujeres, es el cuarto de mayor incidencia (23, 24). Alrededor del mundo, el CC es considerado una de las principales causas de muerte en mujeres en edad fértil, con reportes

anuales de más de 569.847 casos nuevos, de los cuales 311.365 terminan en muertes, ocurriendo el 85% de los casos en países en vías de desarrollo (25).

Para Suramérica se ha reportado una tasa de incidencia de 15,5 casos al año y una tasa de mortalidad del 7,1 casos al año por cada 100.000 mujeres (25). Datos reportados de la incidencia de CC comparado con otros tipos de cáncer en la población femenina, lo sitúan en segundo lugar (antecedido por el cáncer de mama) para algunos países de América del sur; no obstante, para Colombia, esta neoplasia se ubica en primer lugar de aparición en mujeres en edad reproductiva (de 15 a 44 años de edad) (23, 24, 26).

Ha sido ampliamente demostrado que el VPH constituye el factor esencial en el desarrollo del CC. Este tipo de cáncer afecta la porción fibromuscular inferior del útero que se proyecta dentro de la vagina; los dos tipos principales de células que cubren el cérvix son las células escamosas (localizadas en el exocérvix) y las células glandulares (ubicadas en el endocérvix). El punto en el que estos tipos de células se encuentran, se conoce como zona de transformación, que además presenta un constante recambio celular, por lo que es blanco de la infección por ITS como el VPH (27, 28).

De acuerdo a las características histopatológicas, los CC se clasifican en: carcinoma de células escamosas (CCE), el cual es el tipo de presentación más frecuente; el adenocarcinoma (ADC), que compromete células glandulares; y finalmente, el carcinoma adenoescamoso, el cual es el más agresivo y de peor pronóstico (29). Por otra parte, las lesiones precancerosas que progresan a CCE son clasificadas como neoplasia intraepitelial cervical (NIC) o lesión escamosa intraepitelial (LEI), que según el grado de la lesión, pueden ser de bajo grado (NIC1 o LEIBG) o de alto grado (NIC2/3 o LEIAG) (Figura 1) (30).



**Figura 1.** Clasificación de los cambios histológicos en las lesiones precancerosas y cancerosas de CC (31).  
 Abreviaturas: Lesiones Intraepiteliales escamosas de bajo grado (LSIL, de la sigla en inglés: Low-grade squamous intraepithelial lesion); Lesiones Intraepiteliales escamosas de alto grado (HSIL, de la sigla en inglés: High-grade squamous intraepithelial lesion); neoplasia intraepitelial cervical (CIN, de la sigla en inglés: Cervical intraepithelial neoplasia)

Se ha establecido que el CC puede ser considerado como prevenible y, en caso de desarrollarse, si se trata tempranamente es remediable (32). La detección temprana constituye una herramienta útil en la disminución de la carga de la enfermedad, por lo que se han implementado métodos como prueba de Papanicolaou (test Pap) o citología cervical, la cual consiste en la extracción de células de del cérvix para ser visualizadas a través de microscopía. Esta prueba es considerada como un método eficiente de tamización, debido a que permite evidenciar los cambios morfológicos en las células de la zona de transformación del cérvix a un costo significativamente bajo y es de fácil acceso (33).

La prueba de Papanicolaou es actualmente realizada como una herramienta preventiva de CC, sin embargo, presenta mayor apoyo diagnóstico cuando es utilizada en

conjunto con otros métodos de tamización más precisos como son los estudios colposcópicos e histopatológicos. Para la realización de estos métodos, se requiere un examen visual empleando un colposcopio, que es un dispositivo binocular el cual permite iluminación y ampliación del cérvix de seis a cuarenta veces; ésta técnica se debe llevar a cabo por personal médico capacitado para identificar completamente la lesión y proponer simultáneamente el tratamiento (33).

La implementación de esta técnica ha permitido una reducción de las tasas de mortalidad en países desarrollados; sin embargo, en países como Colombia, esta misma tendencia no ha sido la observada (34). Esto se debe a que los programas de tamización implementados en nuestros países, se centran en el cumplimiento del número de citologías realizadas anualmente, faltando aún fortalecer acciones enfocadas en el control de calidad, la adherencia, el seguimiento de la población que asiste al control citológico, el control de la reclamación de los resultados del test Pap, entre otros (35, 36).

Considerando las limitaciones de las estrategias usadas para reducir la carga de CC, en la actualidad se están enfocando los esfuerzos en la identificación de los factores de riesgo asociados, siendo la infección por VPH el principal factor de riesgo de interés para el monitoreo, debido a que ha sido demostrada la relación causal entre las infecciones persistentes y el desarrollo CC (37, 38). Del total de tipos virales descritos, 13 han sido clasificados como de alto riesgo (VPH-AR) dada su capacidad oncogénica, siendo los tipos VPH-16, -18, -31, -33, -35, -45, -52 y -58, los de mayor prevalencia en la población a nivel mundial (39, 40).

En la búsqueda de disminuir la mortalidad por esta neoplasia, han sido incorporados métodos de detección del VPH, que junto con el Pap test buscan identificar mujeres en riesgo de desarrollar neoplasia cervical (41). La implementación conjunta de estos métodos ha mostrado ser eficiente en la detección de lesiones cervicales persistentes, permitiendo la identificación de la enfermedad en estadios tempranos, el incremento entre los tiempos de tamización y el tratamiento oportuno en estadios tempranos de la enfermedad (42). Adicionalmente, conocer el estado de infección por VPH permite una interpretación de la

citología por parte del experto más acertada y sensible, en comparación con aquella lectura donde se desconoce el estado de la infección viral (43).

### ***Virus del Papiloma Humano - VPH***

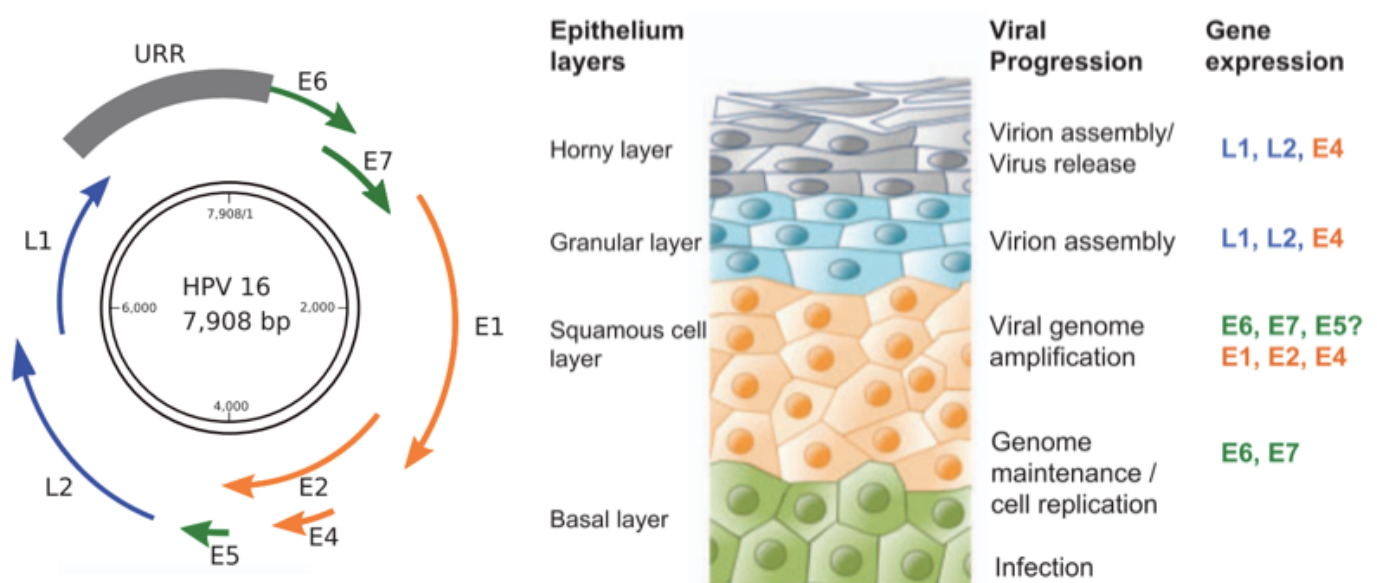
El Virus de papiloma humano pertenece a la familia *Papillomaviridae*; estos virus son de aproximadamente 50 nm y tienen un genoma de ADN de doble cadena circular de 8.000 pares de bases (pb), contenido dentro de una cápside icosaédrica no envuelta (44). Su genoma se divide en tres partes: una región que codifica para las proteínas tempranas no estructurales: E1, E2, E4, E5, E6, E7 (de la sigla en inglés: Early), las cuales regulan la fase vegetativa y productiva del ciclo viral; una segunda región que codifica para las dos únicas proteínas estructurales L1 y L2 (de la sigla en inglés: Late), que constituyen la cápside viral; finalmente, una tercera región reguladora no codificante, denominada LCR (de la sigla en inglés: Long Control Region), implicada en la regulación de la replicación y transcripción viral (45).

La región temprana (E), que compone el 45% del genoma del virus, esta subdividida en dos: por un lado, compuesta por los genes que codifican para las proteínas E1 y E2, encargadas de regular la replicación viral; E1 regula la replicación de ADN, mientras que E2 es un factor de transcripción que interactúa con los motivos ACCN6GGT en la región viral LCR; las proteínas E2 de los VPH-AR tienen la capacidad de actuar como activadores transcripcionales, asociándose con E1; esta interacción es necesaria para el reconocimiento eficiente del origen y replicación del genoma viral (46).

La segunda sub-región E está compuesta por los genes *E5*, *E6* y *E7* que codifican proteínas con alto poder oncogénico, encargadas de la proliferación celular (47). La denominada E5 actúa como proteína transformante, interactuando con los factores de crecimiento celular (48). Las proteínas E6 son pequeñas (de aproximadamente 150 aminoácidos) y contienen dos dominios en los que se incluyen motivos CXXC apareados, cada uno de los cuales está relacionado con el 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 (49, 50).

La región LCR, que compone el 15% del genoma viral, aunque no codifica ninguna proteína, contiene los promotores y los sitios donde inicia la replicación del genoma del virus (49). Finalmente, en la región de proteínas tardías o de envoltura (L), se codifican las proteínas L1 y L2; la L1 es la proteína mayor de la cápside, representando más del 90% de las proteínas del virión y es el principal componente de la superficie del virus. La L1 está implicada en la invasión del virus a las células epiteliales y es un blanco especial para su detección molecular (48). La proteína L2 hace parte de la menor proporción proteica de la cápside, e interactúa con L1 a través de un dominio hidrofóbico próximo al carboxi-terminal (51-53) (Figura 2).



**Figura 2.** Organización del genoma y ciclo de vida del VPH (49).

Los VPH tienen tropismo celular por el epitelio escamoso estratificado e infectan las células epiteliales basales de la unión escamo-columnar del cuello uterino, aunque han sido aislados de otros sistemas como el respiratorio, intestinal y sanguíneo; esta adaptación

evolutiva está asociada a la necesidad de tener una amplia gama de colonización en diversos tejidos corporales que les permita proliferar de manera eficiente (54). El virus hace su entrada en las células epiteliales basales (debido a que éstas se encuentran mitóticamente activas, lo que favorece el ciclo de vida del virus) a través de micro-heridas o microabrasiones, en donde puede permanecer en estado episomal (no integrado) o puede tomar la vía de integrarse al ADN del hospedero; su ciclo de vida se encuentra estrechamente vinculado a la diferenciación del epitelio del hospedero (Figura 2) (50, 55).

La mayoría de las infecciones por VPH son transitorias, con tiempo medio de resolución de 6 a 18 meses. Cerca del 25% de las infecciones no logran resolverse de forma natural, desarrollando LEIBG, de las cuales, cerca del 20% progresarán a LEIAG. El tiempo medio entre la infección viral y la aparición de evidencia microscópica de malignidad, puede ser de hasta 10 años, por lo que del total de mujeres que tienen contacto con el virus, cerca del 0,05% desarrollarán CC si no es detectado y tratado a tiempo; a pesar del reducido porcentaje de mujeres que desarrollan CC y el largo periodo de entre la infección inicial y la aparición de la neoplasia maligna, las tasas de morbi-mortalidad por esta causa siguen siendo elevadas, debido a las limitantes en los sistemas de detección primaria y a la falta de oportunidad de recibir tratamiento en los estadios tempranos de la enfermedad (56-58).

Los VPH han evolucionado lentamente junto con sus anfitriones, generalmente causando infecciones asintomáticas; de los que infectan humanos, se han secuenciado más de 100 diferentes, los cuales se han agrupado en 5 géneros denominados por letras griegas y exhiben asociaciones diferenciales de enfermedad (49). Basados en la secuencia de *LI*, el Comité Internacional de Taxonomía de los Virus (ICTV, de la sigla en inglés: International Committee on Taxonomy of Viruses), ha clasificado los VPH en géneros (60% de similitud en la secuencia de *LI*), especies (similitud del 70%), tipos virales (90% similitud), variantes moleculares (entre 98 a 99% de similitud) y sublinajes (similitud > 99%) (59).

Actualmente, 40 tipos de VPH (pertenecientes al género Alpha-Papillomavirus) han sido descritos como capaces de infectar la región anogenital, clasificados por la Agencia Internacional para la Investigación del Cáncer, de acuerdo a su potencial oncogénico en: virus de alto riesgo (VPH-AR), entre los que se encuentran VPH-AR-16, -18, -31, -33, -35, -39, -

45, -51, -52, -56, -58, -59, -68, -73 y -82; de bajo riesgo, VPH-BR-6, -11, -40, -42, -43, -44, -54, -61, -70, -72 y -81; y de riesgo intermedio, VPH-26, -53 y -66 (39, 60). Estudios epidemiológicos soportan la evidencia que VPH-AR-16 y -18 son los virus de mayor potencial oncogénico, lo que se refleja en su alta frecuencia en LEIAG (40).

Factores del virus podrían favorecer el desarrollo de CC, entre ellos, las mujeres que cursan con infecciones por múltiples tipos de VPH, dado que estos podrían actuar sinérgicamente y favorecer su persistencia en el hospedero (61, 62). Las variantes moleculares intra-típicas exhiben distintas capacidades de oncogenicidad, integración y persistencia, como se ha sugerido para variantes de VPH-16 (63-65). Adicionalmente, la carga viral podría estar influenciando la dinámica de las infecciones virales y la gravedad de la lesión cervical (Figura 3) (66, 67).

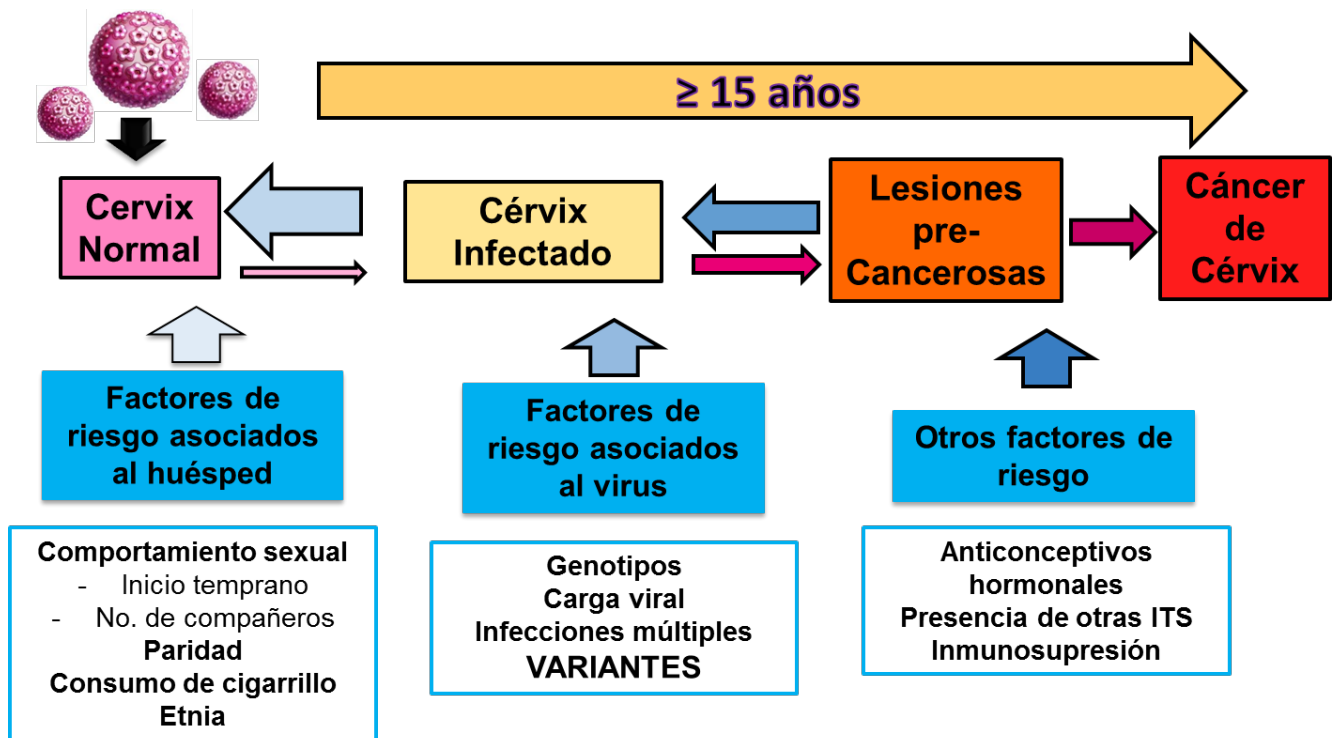


Figura 3. Historia natural de la carcinogénesis cervical (68, 69).

La importancia clínica de las infecciones por múltiples tipos de VPH-AR (coinfecciones) aún es controversial. Tradicionalmente, una lesión es atribuida a la infección por un único tipo de VPH (70). No obstante, estudios han demostrado una alta prevalencia de coinfecciones en lesiones cervicales (LEIBG o de mayor grado), en las cuales atribuir la relación causal a un único genotipo viral se dificulta (70).

Adicionalmente, estudios *in vitro* de la biología de las infecciones múltiples han demostrado la capacidad de una misma célula para albergar más de un tipo de VPH, sin embargo, al evaluar esto en mujeres con lesiones, los resultados son poco concluyentes (70-72). 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, siendo este tipo viral el responsable de los cambios a nivel celular (72).

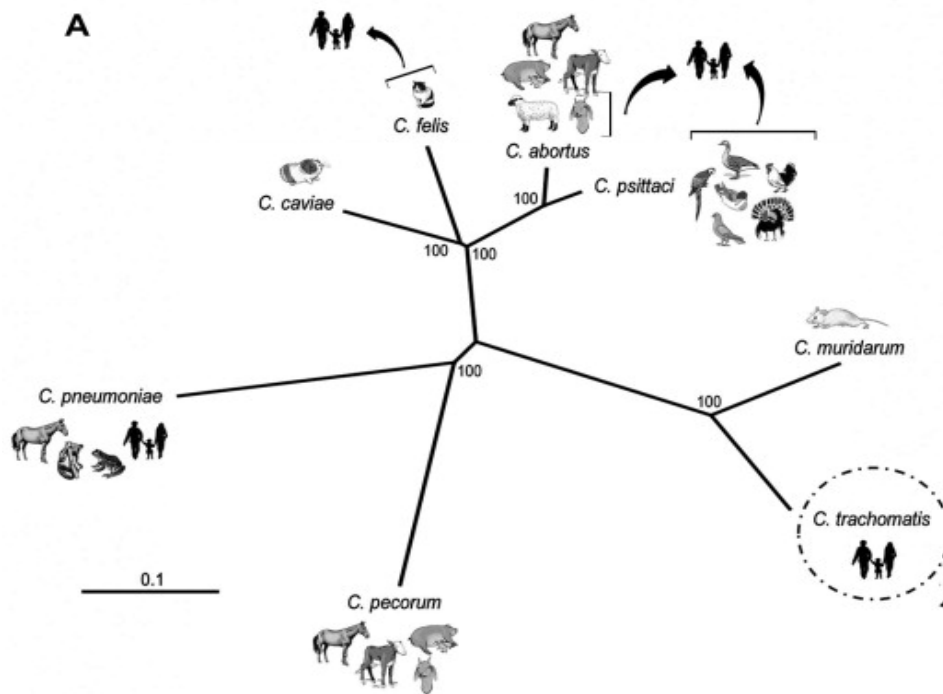
En cuanto a la carga viral, estudios sugieren una relación entre el aumento en el número de copias virales y el grado lesión, principalmente para los tipos VPH-16 y -18 (73, 74). Se ha sugerido que en lesiones cervicales donde se identifica VPH-16, gran cantidad de copias del virus son encontradas, principalmente integradas al genoma del hospedero (75); no obstante, otro estudio reporta que mujeres con menores cargas virales, presentaban mayor persistencia viral y hallazgos citológicos de peor pronóstico (76).

### ***Chlamydia trachomatis***

El phylum *Chlamydiae*, comprende un grupo de bacterias cocoides, no móviles, intracelulares obligadas, que exhiben un característico ciclo de vida bifásico (77, 78). Algunos de los miembros son considerados patógenos emergentes, tanto para humanos, como para animales (79, 80).

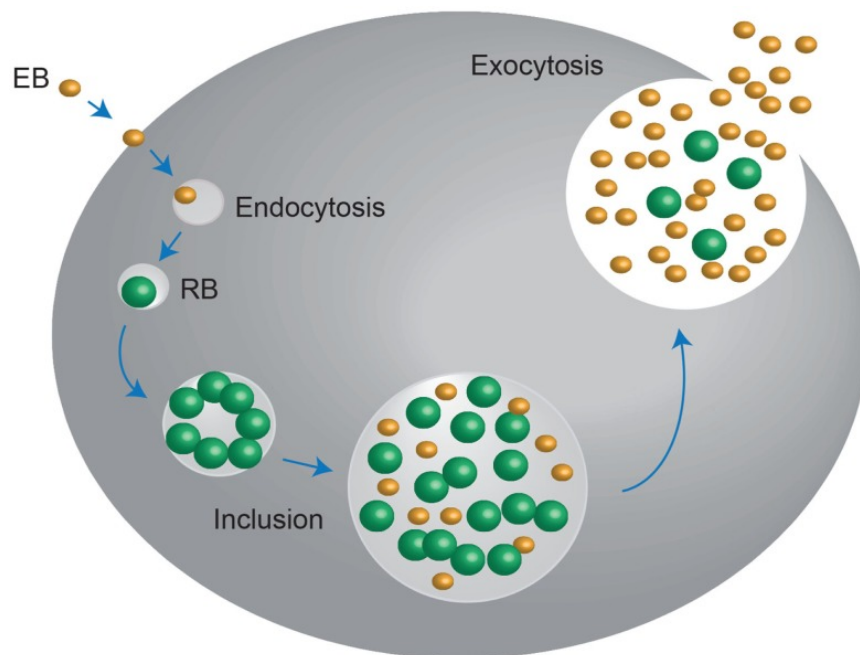
Una de las familias clínicamente más relevantes dentro de este phylum es conocida como *Chlamydiaceae*, la cual está compuesta por once especies (81, 82); algunas especies de esta familia son consideradas patógenos de interés en salud por producir infecciones zoonóticas donde los humanos son hospederos accidentales, como *C. abortus*, *C. psittaci*, *C. pneumoniae*, entre otras (Figura 4) (83, 84). No obstante, *C. trachomatis* constituye la especie

de mayor impacto a nivel de salud pública, siendo considerada una de las infecciones de mayor prevalencia a nivel mundial (85-90).



**Figura 4.** Especies del phylum Chlamydiae y sus hospederos naturales (91).

*C. trachomatis* tiene un ciclo de vida bifásico constituido por cuerpo infeccioso extracelular pero metabólicamente inerte conocido como cuerpo elemental (CE), el cual es responsable de la invasión y la diseminación de la infección a las células vecinas; y un cuerpo reticulado (CR) no infeccioso intracelular, pero metabólicamente activo (78, 79, 92-94). El ciclo de vida inicia con la unión de CE a la célula del hospedero, seguido por la posterior internalización y formación de una vacuola parasitófora derivada de lípidos de la célula hospedera; en esta inclusión, ocurre la diferenciación a CR y su multiplicación por fisión binaria; finalmente, la progenie se diferencia nuevamente a CE que se liberan a través de lisis e infectan nuevos blancos celulares (Figura 5) (93, 95).



**Figura 5.** Ciclo de vida bifásico de *Chlamydia trachomatis* (96). Abreviaturas: Elementary Body (EB o Cuerpo elemental); Reticulate Body (RB o cuerpo reticular).

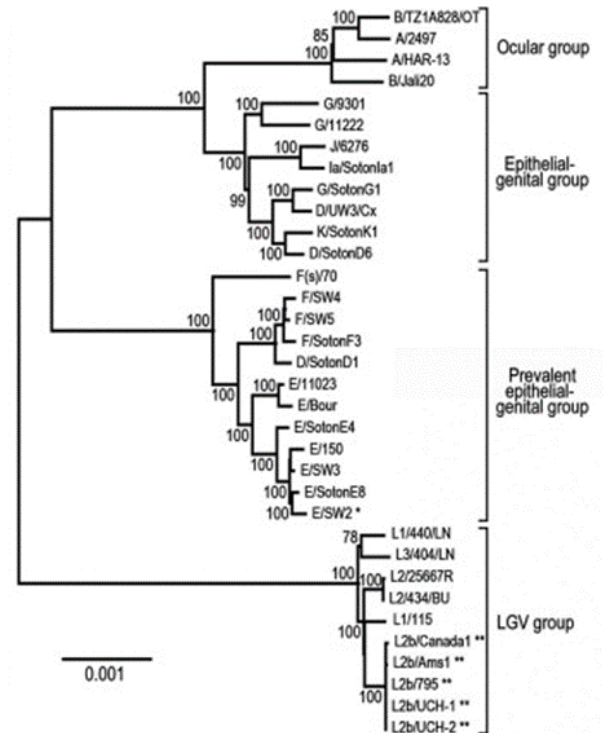
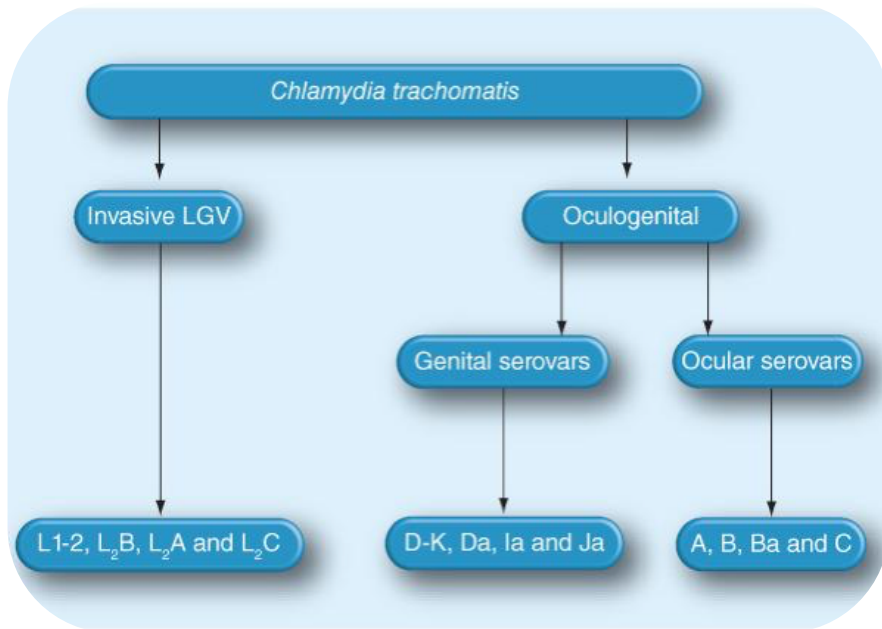
*C. trachomatis* afecta exclusivamente humanos, infectando principalmente el tracto urogenital, representando la ITS de origen bacteriano más común a nivel mundial (97), especialmente en África y en el Medio Oriente, zonas endémicas para esta infección (98). Alternativamente, *C. trachomatis* puede infectar la mucosa ocular, siendo responsable del desarrollo de tracoma, la principal causa de ceguera infecciosa alrededor del mundo (99-102).

Su ciclo de vida bifásico, restringido a ser intracelular obligatorio, le ha traído como consecuencia la pérdida de capacidad de sintetizar nutrientes esenciales como el triptófano (44, 93, 94, 103). Su genoma está compuesto por un único cromosoma circular de más de 1 millón de pb, y un plásmido de ~7,5 kb, el cual es altamente conservado, con ocho marcos de lectura abiertos, y un regulador que controla su número de copias (algunas bacterias tienen hasta 10 copias del plásmido) (91, 104, 105); estos plásmidos son reconocidos como el mayor

factor de virulencia, puesto que participan en la síntesis del glicógeno y codifican proteínas responsables de la virulencia (104-108).

Dado su estricto carácter intracelular, que les permite crecer y sobrevivir en un ciclo de vida bifásico, los Chlamydiales han evolucionado lentamente, caracterizándose por conservar pequeños genomas (de aproximadamente 1,2 Mpb), eventos de origen, duplicación y transferencia de genes en bajas tasas, así como alta conservación génica en términos de contenido y sintenia (definido como las regiones genómicas que se conservan entre múltiples especies) (81, 82, 109, 110).

Hasta el momento, 19 variantes han sido descritas y son clasificadas en dos grandes grupos: las genovariantes L, causantes de linfogranuloma venéreo (LGV); dentro de este grupo han sido descritas L1-L3, L2a-b, L2c, sus infecciones son altamente invasivas, ulcerativas y requieren tratamiento por largos periodos (91, 110, 111). La segunda agrupación, comprende variantes asociadas a infecciones oculo-genitales, las cuales pueden tener un curso clínico asintomático, sin complicaciones y limitadas al tejido escamo-columnar de membranas mucosas; dentro de éstas, las genovariantes A, B, Ba y C se encuentran asociadas principalmente a tracoma, mientras que las D-K, Da, Ia y Ja afectan el tracto urogenital (Figura 6) (3, 112, 113).



**Figura 6.** Clasificación y filogenia de las variantes de *C. trachomatis* según el tropismo tisular (91, 114).

A nivel clínico, las infecciones por *C. trachomatis* pueden derivar en distintas afecciones, dependiendo de la variante y el epitelio afectado; algunas se encuentran asociadas con enfermedades oculares, siendo la principal causa infecciosa de ceguera cuando no se detecta y trata a tiempo, mientras que otras variantes, causan infecciones genitales que pueden resultar en graves consecuencias para la salud reproductiva (94, 103, 115). Estas infecciones son de fácil tratamiento cuando se detectan a tiempo, sin embargo, las limitaciones en el diagnóstico de los programas de salud sexual y reproductiva implementados (principalmente en países en vías de desarrollo), han contribuido a su alta transmisión, la cual va ligada a un comportamiento sexual de riesgo (116).

Una de las principales consecuencia de las infecciones por *C. trachomatis* no detectadas y tratadas, es la contribución de esta ITS bacteriana en la displasia cervical, hallazgo que se ha sugerido desde hace más de 5 décadas; estudios epidemiológicos muestran

un significativo incremento en el riesgo de desarrollar anormalidad cervical en aquellas mujeres que cursan con infecciones por *C. trachomatis*, no obstante, otros estudios no han encontrado dicha asociación (116-119). Aunque los mecanismos involucrados en la patogénesis no son claros aún, estudios longitudinales muestran asociaciones de riesgo entre la presencia bacteriana (principalmente variantes D, G e I) y el desarrollo de CCE; esta misma asociación no es observada en lesiones de peor pronóstico ADC, lo que se explica en que el principal blanco de *C. trachomatis* lo constituyen las células endocervicales (120, 121).

Posibles mecanismos involucrados en la patogénesis de *C. trachomatis* y su contribución en la carcinogénesis podrían incluir liberación de óxido nítrico, procesos inflamatorios, inhibición de la apoptosis, alteración de la estructura, regulación y mantenimiento celular, a través de liberación de especies reactivas del oxígeno, entre otros (119, 122-125). Estos mecanismos, además del daño a nivel celular, favorecen el incremento de carga viral de VPH, la integración del virus al genoma del hospedero y la sobreexpresión de E6/E7-VPH conduciendo al crecimiento y proliferación celular descontrolada (119, 126).

Las infecciones por *C. trachomatis* que afectan el tracto urogenital suelen ser crónicas, con sintomatología solo en cerca del 20% de las mujeres que se infectan (127), con procesos inflamatorios limitados, pero su persistencia en el cérvix conlleva a su hipertrofia y a la inducción de metaplasia (128); este patógeno bacteriano pueden coexistir con VPH, posiblemente con un efecto sinérgico para las dos infecciones (119, 126, 129).

Un estudio *in vitro* demostró que VPH y *C. trachomatis* pueden infectar una misma célula, en donde la expresión de E6-VPH modula el efecto de la privación de triptófano, aminoácido necesario en el crecimiento y replicación de *Chlamydia*; como consecuencia de esta modulación, *C. trachomatis* entra en un estado de silencio antigénico, a través de la reducción de la traducción bacteriana y dirigiéndose hacia un CR no replicativo pero viable y persistente dentro de las células; esta coexistencia (VPH – *C. trachomatis*) facilita y promueve la supervivencia bacteriana hasta que disminuya la respuesta inmune del hospedero, por lo que la presencia de VPH proporciona condiciones favorables para la supervivencia de infecciones por *C. trachomatis* (103).

Diferentes técnicas de diagnóstico de *C. trachomatis* han sido implementadas, las cuales en general han permitido determinar el tropismo tisular de las cepas, identificar y diferenciar infecciones persistentes, entender la dinámica de transmisión y realizar vigilancia epidemiológica de cepas específicas (130). La técnica tradicionalmente utilizada para la tipificación de *C. trachomatis* ha sido la serotipificación, la cual se basa en la identificación, mediante anticuerpos específicos, de la proteína de la membrana externa MOMP; dada la necesidad del cultivo celular para la propagación de la bacteria, esta técnica resulta laboriosa, consume demasiado tiempo y presenta una baja sensibilidad (91, 131).

Para la tipificación de las distintas variantes, técnicas moleculares han sido desarrolladas, dentro de las cuales se describen los polimorfismos de longitud de fragmentos de restricción o RFLP (de la sigla en inglés: Restriction Fragment Length Polymorphism), técnicas basadas en hibridación del ADN, microarreglos y secuenciación. Muchas de estas técnicas se dirigen hacia la detección y análisis del gen *ompA* (el cual codifica para la proteína MOMP) (111, 132-137).

Debido al bajo poder de discriminación de algunas de las técnicas anteriormente descritas y a las múltiples desventajas que presentan, estas han sido remplazadas por otros métodos de tipificación, tales como el análisis de repeticiones en tándem de un número variable de loci - MLVA (de la sigla en inglés: Multiple loci VNTR analysis) y la tipificación de secuencias multilocus - MLST (de la sigla en inglés: Multilocus sequence typing) (89, 107, 137-139); estas técnicas han mostrado ser mucho más específicas, además de permitir una mejor tipificación a nivel de taxa, especie y variante (107).

### ***Virus de la Inmunodeficiencia Humana – VIH***

El Virus de la Inmunodeficiencia Humana (VIH), descrito por primera vez en 1981, ha sido incluido dentro de la familia Retroviridae, género Lentivirus, debido a sus características biológicas, morfológicas y genéticas. Este virus es responsable de alrededor de 75 millones de infecciones y 25 millones de muertes al año a nivel mundial (140, 141). En Colombia, se reporta el 10% de los casos de Latinoamérica y se estima que más 150.000 personas están infectadas con el VIH en nuestro país (142). El VIH tiene tropismo por las

células del sistema inmune, como los macrófagos, células dendríticas y células T CD4+, siendo estas últimas el principal objetivo del virus, donde se replica para invadir otras células del hospedero (141).

El VIH se clasifica en tipos (VIH-1 y VIH-2), grupos, subtipos y formas recombinantes (143). El VIH-2 se encuentra limitado a algunas zonas de África Central y Occidental, mientras que el VIH-1 se ha aislado en diferentes partes del mundo y es considerado como el más virulento y patógeno (141); para este tipo, cuatro grupos han sido descritos: M, O, N y P. El tipo M está estrechamente relacionado con el virus de inmunodeficiencia del simio y se encuentra ampliamente distribuido, causando más del 90% de las infecciones. Nueve subtipos (clados) comprenden este grupo: A-D, F-H, J y K; de estos subtipos, el B se encuentra con mayor frecuencia en países desarrollados (como Australia y países Europeos), mientras que los subtipos C y D son más agresivos y presentan un progreso de la enfermedad acelerado; finalmente, hasta el momento más de 50 formas recombinantes circulantes han sido descritas para VIH-1 (144, 145).

El VIH está constituido por un genoma de ARN de cadena sencilla lineal dimérica de sentido positivo, que cuenta con nueve genes que se transcriben policistricamente y que van dando origen a distintas proteínas, entre los que se encuentran tres principales: *gag* (de cápside), *pol* (para la replicación) y *env* (glicoproteína externa de unión a receptores de la célula hospedera); adicionalmente, están los genes que codifican para proteínas estructurales: matriz o *p17*, cápside o *p24*, nucleocápside o *p9* y *p6*; los genes que codifican para enzimas: proteasa, transcriptasa inversa e integrasa; para las proteínas de la envoltura: GP120 y GP41; y finalmente los genes reguladores *Tat* y *Rev* y proteínas accesorias: Vif, Vpr, Vpu/Vpx y Nef (Figura 7) (146, 147).

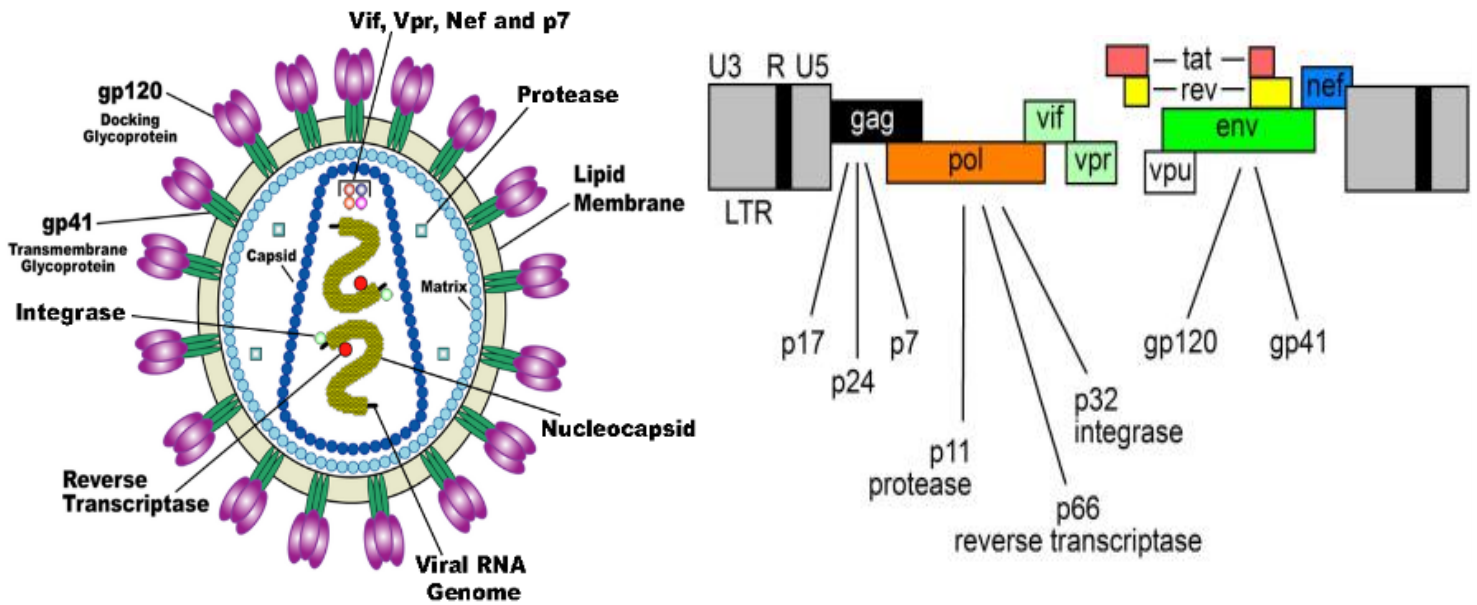


Figura 7. Estructura y organización del genoma del VIH (148).

El ciclo de vida viral inicia con el reconocimiento por parte de gp120 y gp41 a la glicoproteína CD4 de las células T, permitiendo la unión, entrada y fusión de las membranas del VIH y la célula hospedera; a continuación, el ARN del virus se transcribe a ADN por acción de la transcriptasa inversa; este ADN se internaliza en el núcleo de la célula CD4, donde ayudado por la integrasa viral, se integra al genoma del hospedero (allí puede permanecer en estado de provirus durante algún tiempo); posteriormente, se transcriben proteínas celulares junto con las virales y son exportadas fuera del núcleo; finalmente, las proteínas estructurales del virus sintetizadas son ensambladas y liberadas de la célula hospedera (llevando parte de la envoltura exterior), donde la proteasa viral actúa mediando la maduración hacia una partícula viral infecciosa (141, 149, 150).

El VIH constituye una de las causas más frecuentes de inmunosupresión en la población y conduce a la alteración de la inmunidad mediada por células, facilitando la adquisición de infecciones oportunistas y limitando al organismo para establecer respuestas

inmunes eficientes para hacer frente a procesos patológicos (151). ITS como *C. trachomatis* pueden aumentar la probabilidad de adquirir y transmitir la infección por el VIH (152). No obstante, una de las infecciones concomitantes más comunes es la que ocurre con el VPH; se ha documentado que en mujeres que conviven con VIH, se presenta una elevada prevalencia de infección por VPH, con menores tasas de depuración (153, 154).

Las mujeres con conteos de células CD4 menores de 200 células/mm<sup>3</sup> presentan mayores prevalencias de infección con VPH e incremento en las tasas de incidencia de lesiones intraepiteliales cervicales, en comparación con las mujeres con recuentos de CD4 mayores de 500 células/mm<sup>3</sup>, lo cual, sumado a una alta carga de viral (medida en número de copias de VIH), podría incrementar 10 veces el riesgo de desarrollar este tipo de lesiones (155), razón por la cual, este tipo de cáncer está catalogado como el más frecuentemente diagnosticado en mujeres infectadas con VIH (153, 156).

Teniendo en cuenta que estas dos infecciones son transmitidas sexualmente, la adquisición de VPH se ve favorecida por el impacto en la inmunidad derivada de la seroconversión de VIH, además, la inmunosupresión contribuye a la reactivación de infecciones por VPH en estado latente, así como a la persistencia de éstas a nivel del cérvix. Aunque los dos virus pueden llegar a ser detectados en el epitelio cervical de zonas con lesiones, estos no tienen tropismo por el mismo tipo de células, por lo que la coexistencia de estos patógenos en un mismo hospedero es atribuida a interacciones indirectas (157, 158). A nivel molecular, se ha demostrado un incremento en la transcripción de los genes E6/E7 en el VPH, mediada por la expresión de tat y gp120 en el VIH (159, 160). También ha sido descrita la activación de la replicación del VIH, como consecuencia de la producción de citoquinas (como interleuquina-6 (IL-6)) por parte de células cervicales infectadas por VPH (161).

## OBJETIVOS

### ***Objetivos generales***

#### *Componente Longitudinal - Cohorte*

Determinar la incidencia de infección por *C. trachomatis* y su relación con la presencia y carga viral de seis tipos de Virus del Papiloma Humano de alto riesgo en una cohorte de mujeres colombianas.

#### *Componente de corte Transversal*

Establecer la frecuencia de la infección, distribución tipo-específica y coinfección de seis tipos de Virus del Papiloma Humano de Alto Riesgo en un grupo de mujeres colombianas con y sin infección por Virus de Inmunodeficiencia Humana.

### ***Objetivos específicos***

#### *Componente Longitudinal - Cohorte*

1. Describir la dinámica de la carga viral de seis tipos de VPH de alto riesgo en las mujeres que conforman la cohorte.
2. Determinar la frecuencia de *C. trachomatis* y establecer los tiempos de duración de las infecciones en la población de estudio.
3. Comparar la incidencia de infección por *C. trachomatis* y su relación con la prevalencia y carga viral de seis tipos de VPH-AR.
4. Evaluar la eventual asociación entre infecciones por VPH, *C. trachomatis* y los factores de riesgo de la población de estudio.

#### *Componente de corte Transversal*

5. Establecer la frecuencia infección única, múltiple y la distribución tipo-específica de seis tipos de VPH en mujeres con y sin infección por HIV.

6. Analizar la eventual asociación entre las infecciones por múltiples tipos de VPH y el status de infección por VIH en las mujeres del estudio.

## INTRODUCCIÓN A LOS CAPÍTULOS

La relación causal entre la infección por VPH-AR y el desarrollo de CC ha sido ampliamente demostrada (162, 163). A pesar de la elevada frecuencia de infección por este virus, una gran fracción de las infecciones se resuelven espontáneamente (entre 6 y 18 meses), permaneciendo una reducida parte de ellas de manera persistente (164-167). Dentro de los distintos factores que contribuyen a persistencia de VPH-AR, la carga Viral (CV) parece tener un rol esencial en el mantenimiento del virus en el organismo (165, 167-170); la evidencia publicada sugiere una relación entre el aumento de copias de VPH-AR y la presencia de lesiones a nivel cervical (73, 171, 172).

Por el contrario, otros estudios indican que son las infecciones virales con baja carga las que contribuyen a la proliferación anormal de la células, puesto que estas infecciones no son detectadas por el sistema inmune, lo que permite la presencia del virus en el epitelio cervical por largo tiempo (170, 173). Si bien el rol de la CV como marcador de progresión de las lesiones precancerosas sigue siendo controvertido, dichas discrepancias pueden en parte ser explicadas por factores como el tipo de VPH-AR responsable de la infección (38, 170, 174) y la coexistencia con otras ITS (175, 176).

Las ITS son una causa importante de morbilidad y un problema de salud pública a nivel mundial (177, 178). Infecciones persistentes por algunas de éstas provocan daño progresivo del cérvix y han sido asociadas como cofactores en la patogénesis de CC (178, 179). En los últimos años, las infecciones concomitantes con VIH y *C. trachomatis*, han sido relacionadas con la dinámica de las infecciones por VPH-AR y como cofactor en el progreso a lesiones cervicales (179-186).

En la Fundación Instituto de inmunología de Colombia (FIDIC), desde el año 2006, se viene desarrollando un estudio multicéntrico que ha permitido realizar el análisis epidemiológico de la infección por Virus de Papiloma Humano (VPH) en la población colombiana. Este estudio ha incluido cerca de 50,000 mujeres de 15 departamentos y 32 instituciones colaboradoras para su ejecución. Se ha logrado establecer que en Colombia, en la población estudiada, cerca del 50% de las mujeres cursa con infecciones por VPH, coincidentemente en las regiones que presentan las mayores tasas de morbilidad y mortalidad

asociadas a CC y que presentan acceso limitado a servicios de salud, entre otros factores de riesgo.

Como resultados de este estudio multicéntrico, se encontró que el VPH-16 ocupa primer lugar en frecuencia en las regiones analizadas, seguido por VPH-31, siendo la distribución tipo-específica variable según la región geográfica analizada (187). Adicionalmente, a través de un estudio longitudinal, se evaluó la dinámica de las infecciones por seis tipos de VPH-AR (VPH-16, -18, -31, -33, -45 y -58) en mujeres provenientes de tres ciudades: Chaparral (localizado en el departamento del Tolima), Girardot (ubicado en el departamento de Cundinamarca) y Bogotá (Distrito Capital de Colombia). Este estudio, fue pionero en nuestro país, ya que además de incluir población heterogénea (en términos socioeconómicos y geográficos, entre otros), se contó con la detección y cuantificación (por PCR cuantitativa en tiempo real - qPCR) de tipos de VPH-AR diferentes a VPH-16 y -18. Los resultados mostraron que VPH-18 y -31 fueron los tipos virales de mayor persistencia y menor probabilidad de resolver la infección en el tiempo evaluado, en contraste, los tipos VPH-16 y -33, mostraron menores tiempos de depuración; la persistencia viral y el empeoramiento de los hallazgos citológicos se relacionaron con las infecciones con menores cargas virales (para VPH-16, -31 y -58); finalmente, para el tipo VPH-45, el número de copias influyó en la probabilidad de eliminación de su infección (76).

Si bien, con este primer estudio se dieron avances en la comprensión de la dinámica de las infecciones virales a través del tiempo, quedaron preguntas por resolver como el potencial uso de la carga viral como predictor del desenlace de las infecciones por VPH y la influencia de la CV en la coexistencia con otras ITS. Teniendo en cuenta que, de las distintas ITS descritas en los últimos años, las infecciones concomitantes con *C. trachomatis* han sido foco de estudio y se han descrito como un cofactor clave en el progreso de las lesiones cervicales (179-184), se planteó el **Capítulo 1** de esta tesis, con el fin de determinar el comportamiento de la carga viral de acuerdo al estado del cérvix (indicado por el resultado de la colposcopia) y la infección por *C. trachomatis*.

Para este primer capítulo, se continuo con el estudio longitudinal, en el que se habían colectado muestras cervicales e información de factores de riesgo a mujeres colombianas; el

seguimiento fue de al menos dos años y se contó con visitas programadas con periodicidad 6 meses ( $\pm$  3 meses) entre visitas. En cada ocasión, se llevó a cabo la detección, tipificación y cuantificación (por qPCR) de 6 tipos de VPH-AR (16, -18, -31, -33, -45 y -58) y la detección de *C. trachomatis* por PCR convencional.

Las frecuencias en la detección de VPH a lo largo del seguimiento variaron entre 70,6 a 79,2%; a nivel tipo específico, el VPH-16 fue el de mayor frecuencia en la línea base, con disminución estadísticamente significativa de su prevalencia a lo largo de los seguimientos. Para evaluar la dinámica de la carga viral (de los 6 tipos virales detectados) y su relación con el hallazgo colposcópico, se agruparon las mujeres en dos grupos. En el primer grupo las mujeres con LEI, incluyendo mujeres cuya colposcopia reportaba lesiones intraepiteliales de bajo o alto grado; y en el segundo grupo, mujeres sin LEI, las cuales tenían una colposcopia negativa para lesiones intraepiteliales. Cada mujer con lesión fue emparejada con dos mujeres sin LEI (razón entre los dos grupos de 1:2), teniendo en cuenta la edad y la fecha de ingreso al estudio de cohorte. Los resultados mostraron asociaciones para VPH-16, donde en mujeres con baja carga viral, la presencia de lesiones fue más frecuente, mientras que para VPH-31, aquellas con alta carga viral se asociaron con una menor frecuencia de LEI. Estos resultados sugieren la asociación entre la carga del VPH y la frecuencia de anomalía cervical a nivel tipo-específico y contribuyen al entendimiento del efecto de la carga viral en la historia natural del CC.

Para evaluar la relación entre la presencia de VPH y *C. trachomatis*, así como el efecto de la carga viral en la infección, depuración y persistencia bacteriana, se determinó la prevalencia de *C. trachomatis* en aquellas mujeres de la cohorte que iniciaron con infección por VPH, la cual resultó positiva para una cuarta parte de la población y fue incrementándose a lo largo de los seguimientos.

En cuanto a los factores de riesgo asociados a la infección por *C. trachomatis*, los resultados mostraron una mayor probabilidad en aquellas mujeres con infección simultánea por más de un tipo de VPH (definida para esta tesis como coinfección), mayor número de compañeros sexuales y antecedentes de abortos; en contraste, se observó una menor probabilidad de infección bacteriana en mujeres con uso de anticonceptivos hormonales como método de planificación. Por otra parte, se encontró que mujeres con antecedentes de

otras ITS, presentaron mayor probabilidad de eliminación de *C. trachomatis*. Finalmente, en cuanto a la persistencia, se encontró una menor probabilidad de ocurrencia de este evento en mujeres mayores de 45 años.

En cuanto a la carga viral, en la que además se incluyeron mujeres que al inicio de la cohorte no presentaron infección por VPH, los resultados mostraron que las variaciones en la carga viral tenían un efecto en el curso clínico las infecciones bacterianas, por ejemplo, mujeres con número de copias categorizadas como media (entre  $10^0$  a  $10^2$  copias de VPH), presentaron una mayor probabilidad de tener infecciones por *C. trachomatis*; otras asociaciones fueron observadas, siendo dependientes del tipo de VPH infectante.

Por último, dentro de este capítulo, se llevó a cabo un trabajo adicional; teniendo en cuenta que en la literatura se ha reportado que otro factor asociado al riesgo de lesiones cervicales es la genovariante de *C. trachomatis* en infección (188-190), es relevante realizar dicha identificación; en este contexto, se revisó los métodos apropiados con este fin, siendo el sistema de tipificación de secuencias multilocus (MLST) una herramienta molecular que permite además de la tipificación, el establecimiento de la estructura poblacional.

Para *C. trachomatis* se han diseñado diferentes esquemas de genotipificación basados en MLST, sin embargo, a la fecha ninguno de ellos se define como esquema estandarizado y único, y se observa una falta de estudios dirigidos a evaluar la eficiencia de tipificación y discriminación de los marcadores incluidos en los esquemas existentes, que permitan una adecuada clasificación; el objetivo de este trabajo fue, por ende, determinar la robustez de los esquemas y marcadores moleculares usados para la tipificación de esta especie, y la aplicabilidad de estos datos para describir de la variabilidad genética intraespecífica. Para esto, se evaluaron los cuatro esquemas disponibles en el sitio web de Chlamydiales MLST (<https://pubmlst.org/chlamydiales/>): Chlamydiales, *C. trachomatis*, *C. trachomatis*-Uppsala y plasmid loci. Los resultados mostraron un poder de discriminación variable, siendo el esquema de Uppsala el de mayor sobreestimación. Al evaluar los marcadores a nivel individual, se identificaron 8 candidatos, de los cuales 3 (*gidA*, *leuS* y *lysS*) ofrecieron la mayor eficiencia de tipificación, un buen poder de discriminación y se proponen como una alternativa para establecer un esquema único que se desarrolle y valide para este fin.

Como productos del **capítulo 1**, se adjuntan los siguientes artículos científicos:

1. Del Río-Ospina, L; Soto-De León, SC; **Camargo, M**; Sánchez, R; Moreno-Pérez, DA; Perez-Prados, A; Patarroyo, ME and Patarroyo, MA.  
The DNA load of six high-risk Human Papillomavirus types and its association with cervical lesions.  
**BMC Cancer. 2015; 15:100.**
2. Quinónez-Calvache, EM; Ríos-Chaparro, DI; Ramírez, JD; Soto-De León, SC; **Camargo, M**; Del Río-Ospina L; Sánchez, Ricardo; Sánchez, Patarroyo, ME and Patarroyo, MA.  
*Chlamydia trachomatis* Frequency in a Cohort of HPV-Infected Colombian Women.  
**PLoS ONE. 2016; 11(1): e0147504.**
3. Patiño LH; **Camargo M\***; Munoz M; Ríos-Chaparro DI; Patarroyo MA; Ramírez JD.  
Unveiling the Multilocus Sequence Typing (MLST) schemes and Core Genome phylogenies for genotyping *Chlamydia trachomatis*.  
**Front. Microbiol. 2018; 9:1854. \*Primer autor compartido.**
4. **Camargo M**; Sánchez R; Báez-Murcia IM; Soto-De León SC; Patarroyo ME and Patarroyo MA.  
The effect of six types of high-risk human papillomavirus viral load on *Chlamydia trachomatis* infection, clearance and persistence. 2020.  
**Sometido a Front Microbiol. (23-VI-2020).**

Por otra parte, se ha reportado que factores relacionados a la inmunosupresión ocasionada por VIH se asocian con lesiones de mal pronóstico e infecciones por VPH, razón por la cual se propuso analizar, dentro del **Capítulo 2**, la coexistencia VPH-VIH en población colombiana; este tema es de gran relevancia para la priorización de políticas de salud. Para este capítulo, se planteó un estudio de tipo transversal con el fin de establecer la frecuencia de la infección, distribución tipo-específica y coinfección de seis tipos de VPH-AR en un grupo de mujeres colombianas con y sin infección por VIH. Los resultados mostraron frecuencias de infección (69,0%) y coinfección (78,5%) significativamente mayores en mujeres que conviven con VIH, comparadas con aquellas mujeres negativas para VIH

(44,3% y 51,3%, respectivamente). Adicionalmente, un análisis de regresión logística nominal mostró que mujeres VIH positivas tenían 2,37 veces más probabilidad de presentar infección por VPH y 3,43 veces más de tener coinfección. Factores adicionales asociados a las infecciones por VPH, fueron mujeres VIH positivas con conteo de CD4 menor a 500 células/mm<sup>3</sup>, carga de VIH mayor a 100.000 copias/mL y tratamiento con terapia antirretroviral.

Teniendo en cuenta la elevada frecuencia de los eventos de infecciones simultáneas por múltiples tipos de VPH, se llevaron a cabo dos análisis: el primero, que busco establecer asociaciones entre parejas de tipos de VPH de acuerdo al estado de infección por VIH (positivas o negativas). En mujeres VIH negativas, los resultados mostraron fuertes asociaciones entre las parejas de tipos de VPH, sin que la relación filogenética entre tipos virales influyese en esta asociación; en contraste, en mujeres que conviven con VIH, se observaron menos asociaciones entre las combinaciones de genotipos de VPH. Adicionalmente, para evaluar qué factores podrían estar relacionados con el aumento del número de tipos de VPH, se llevó a cabo una regresión logística ordinal. Los resultados mostraron que la probabilidad de coinfección en las mujeres VIH positivas era mayor para aquellas con factores como gestaciones, uso de dispositivo intrauterino, con antecedentes de abortos y cargas virales de VIH >100.000 copias/mL, mientras que en mujeres con conteos >500 células CD4/mm<sup>3</sup> se observó una menor probabilidad.

Como productos del **capítulo 2**, se adjuntan los siguientes artículos científicos:

5. **Camargo M**; Soto-De León SC; Munoz M; Sánchez R; Peña-Herrera D; Pineda AC; Perez-Prados A; Patarroyo ME and Patarroyo MA.  
Human Papillomavirus detection in women with and without Human Immunodeficiency Virus infection in Colombia.  
**BMC Cancer. 2014; 14:451.**
6. **Camargo M**; Del Río-Ospina L; Soto-De León SC; Sánchez R; Pineda AC; Sussmann O; Patarroyo ME and Patarroyo MA.  
Association of HIV status with infection by multiple HPV types.  
**Trop Med Int Health. 2018;23(11):1259-1268.**

***CAPÍTULO 1***  
***COMPONENTE LONGITUDINAL - COHORTE***

RESEARCH ARTICLE

Open Access

# The DNA load of six high-risk human papillomavirus types and its association with cervical lesions

Luisa Del Río-Ospina<sup>1,2</sup>, Sara Cecilia Soto-De León<sup>1,3</sup>, Milena Camargo<sup>1,3</sup>, Darwin Andrés Moreno-Pérez<sup>1,3</sup>, Ricardo Sánchez<sup>1,4</sup>, Antonio Pérez-Prados<sup>5</sup>, Manuel Elkin Patarroyo<sup>1,4</sup> and Manuel Alfonso Patarroyo<sup>1,2\*</sup>

## Abstract

**Background:** Analysing human papillomavirus (HPV) viral load is important in determining the risk of developing cervical cancer (CC); most knowledge to date regarding HPV viral load and cervical lesions has been related to HPV-16. This study evaluated the association between the viral load of the six most prevalent high-risk viral types in Colombia and cervical intraepithelial neoplasia (CIN) frequency.

**Methods:** 114 women without CIN and 59 women having CIN confirmed by colposcopy, all of them positive by conventional PCR for HPV infection in the initial screening, were included in the study. Samples were tested for six high-risk HPV types to determine viral copy number by real-time PCR. Crude and adjusted odds ratios (OR<sub>a</sub>) were estimated for evaluating the association between each viral type's DNA load and the risk of cervical lesions occurring.

**Results:** The highest viral loads were identified for HPV-33 in CIN patients and for HPV-31 in patients without lesions (9.33 HPV copies, 2.95 interquartile range (IQR); 9.41 HPV copies, 2.58 IQR). Lesions were more frequent in HPV-16 patients having a low viral load (3.53 OR<sub>a</sub>, 1.16–10.74 95%CI) compared to those having high HPV-16 load (2.62 OR<sub>a</sub>, 1.08–6.35 95%CI). High viral load in HPV-31 patients was associated with lower CIN frequency (0.34 OR<sub>a</sub>, 0.15–0.78 95%CI).

**Conclusions:** An association between HPV DNA load and CIN frequency was seen to be type-specific and may have depended on the duration of infection. This analysis has provided information for understanding the effect of HPV DNA load on cervical lesion development.

**Keywords:** Cervical intraepithelial neoplasia, HR-HPV, HPV DNA load, RT-PCR

## Background

The main factor for developing cervical cancer (CC) lies in persistent infection by at least one viral type of high-risk human papillomavirus (HR-HPV). Fifteen types of HR-HPV have been described, 99.7% being associated with cases of CC and/or cervical intraepithelial neoplasia (CIN) [1-3]. However, some host and virus related factors modulate such association, i.e. HPV viral load [4,5].

Researchers have thus become interested in HPV viral load. Its association with infection duration has already been described [6,7]. Prior studies have determined the association between viral load and CC severity, progression and development, whilst others have found that the

amount of HPV DNA increases proportionally with lesion severity and can even be detected before cervical lesions develop [8-11]. However, other studies have found no such association [12-14].

As HPV-16 is the viral type most associated with cases of CC (50%–70%) [3,5], most knowledge concerning HPV viral load and CC has been based on HPV-16. Studies, which have included other HR-HPV types, have not led to comparable results regarding those obtained for HPV-16 [15,16].

The real-time polymerase chain reaction (RT-PCR) has been widely used and described in detecting and typing HPV, as well as quantifying a broad range of viral copies and normalising viral load according to the amount of human DNA, having high reproducibility, sensitivity, specificity and yield [13,17]. It was thus considered that it would provide a suitable approach for

\* Correspondence: mapatarr.fidic@gmail.com

<sup>1</sup>Molecular Biology and Immunology Department, Fundación Instituto de Inmunología de Colombia (FIDIC), Carrera 50#26-20, Bogotá, Colombia

<sup>2</sup>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

measuring HPV viral load, thereby facilitating investigating the role of HR-HPV viral load in developing CC [10,12,18].

The present study was thus aimed at using RT-PCR for determining the association between HPV viral load and the presence of CIN for six HR-HPV types, which have been previously reported as having the greatest prevalence in Colombia [19]. It was thus expected to contribute towards knowledge regarding the parameters leading to identifying HPV positive women having a higher risk of developing cervical lesions.

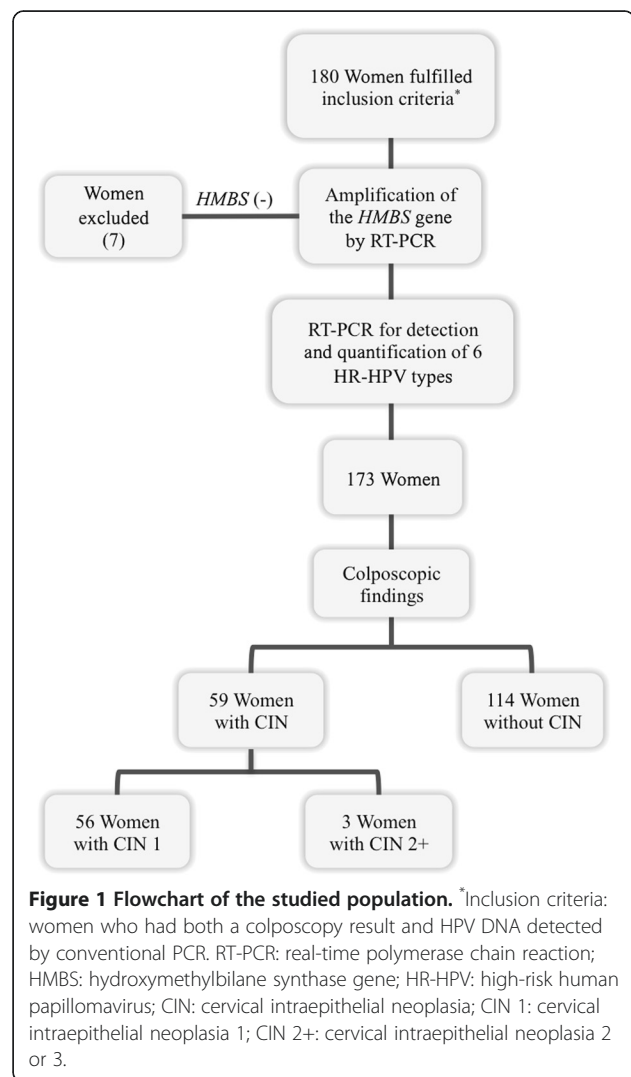
## Methods

### Study population and ethical considerations

Women eligible for the present study were voluntarily attending their cervical screening consultations between April 2007 and March 2010 in three Colombian regions (Girardot, Chaparral and Bogotá). Bogotá (the capital of Colombia) has the highest percentage of inhabitants, being mainly an urban population. Girardot is a city located in the Cundinamarca department which has focused its economy on the tourist sector due to its climate and infrastructure. The city of Chaparral (Tolima department) was included in the study as it is located in Colombia's coffee-growing region and is also known for ecotourism. Girardot and Chaparral were grouped together in the "other city" category to improve the quality of the present study's statistical analysis.

All the 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. Samples were analysed using the Papanicolaou test and HPV DNA detection. Colposcopy and biopsy were performed in accordance with current Colombian screening programme guidelines, thereby establishing that women having normal, satisfactory cytology would continue following the 1-1-3 scheme, meaning that they should have a new control in a year's time and, if this continued being normal, in three year's time. However, colposcopy would be required when cytology was abnormal and, in case colposcopy was abnormal, samples would then be taken for pathology study, as in this study, for diagnosing CIN 1 and CIN 2+ [20]. Colposcopy and biopsy were also carried out for women having normal cytology but who were positive for HPV by conventional PCR, as previous studies have reported an increased risk of CIN 2+ development in women having normal cytology when they are HPV positive [21]. Due to biopsy not being taken from women having negative colposcopy, complete or satisfactory colposcopy (squamocolumnar junction completely visible), evaluation of the transformation area, having normal vascularisation and squamous, cylindrical epithelia without alterations were taken as criteria for guaranteeing the absence of lesions [22].

Colposcopy was chosen as the best method for defining the presence or absence of cervical lesions, as previous studies have found that colposcopy has a good correlation with histological results [23] and it remains the standard for detecting cervical lesions until new methods can be applied; in addition, cervical cytology has been reported worldwide as having variable sensitivity for detecting pre-neoplastic lesions and is considered a screening method which identifies women at risk of developing CC who must then be submitted to definitive diagnostic methods (colposcopy and biopsy) [20,24-26]. Women who had both a colposcopy result and HPV DNA detected by conventional PCR were thus included. Women were excluded in whom there was no amplification of the *Homo sapiens hydroxymethylbilane synthase* (HMBS) gene (Gene ID: 3145) by RT-PCR and those having an insufficient sample for analysis (Figure 1).



This study was supervised and approved by each institution's Ethics Committee as follows: Fundación Instituto de Inmunología de Colombia's Ethics Committee and the Ethics Committee of the Nuevo Hospital San Rafael E.S.E, Girardot, the Hospital San Juan Bautista de Chaparral E.S.E. Bioethics Committee and Hospital de Engativá (level II) Ethics Committee.

#### HPV DNA collection, processing and detection by conventional PCR

Genomic DNA from cervical samples (stored at 4°C, in 95% ethanol) taken from HR-HPV 16, 18, 31, 33, 45 and 58 patients, which had been previously confirmed by conventional PCR (proving positive for at least one of the following previously described primers: GP5+/6+, MY09/11 or pU1M/2R) [27], was extracted using a Quick Extract DNA Extraction Solution kit (Epicentre, Madison, WI), according to the manufacturer's recommendations. The samples were homogenised in 200 µL lysis buffer and incubated at 65°C for 6 minutes and then at 92°C for 2 minutes. The samples were then spun at 13,000 rpm for 10 minutes and the supernatant was stored at -20°C until use.

#### Viral load quantification by RT-PCR

The methodology used in this study has already been described in detail in a previous article by our group [28]. Briefly, specific primers for each viral type and for *HMBS* were synthesised according to a study published by Moberg *et al.* [13]. The probes for each viral type and *HMBS* were designed, taking into account the types included in each reaction. Four parallel duplex real-time PCRs per patient were carried out (Table 1).

The cervical samples processed and identified as being HPV-positive by conventional PCR were used as template in PCR reactions for each fragment. 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 were incubated in LB broth and kept overnight (250 rpm at 37°C). Recombinant plasmids were purified using an UltraClean mini plasmid prep kit (MO BIO laboratories, California, USA) and sequenced using an automatic ABI PRISM 310 Genetic Analyser (PE Applied Biosystems, California, USA). Each insert's integrity was checked by aligning the products with the respective theoretical sequenced fragments from each gene using Clustal W software [29].

#### Real-time PCR

Standardised RT-PCR assays with 10-fold serial plasmid dilutions ( $10^{11}$ - $10^6$  copies) (using known DNA concentration and copy number) gave a standard curve for each viral type and the *HMBS* gene. CFX96 Touch RT-PCR detection system was used for analysis. Samples were tested for HPV-16, HPV-18, HPV-31, HPV-33, HPV-45 and HPV-58. The human *HMBS* gene was amplified in all samples to verify DNA integrity and determine viral copy number per cell. Four RT-PCR reactions were carried out per sample: HPV-16, HPV-18 and -31, HPV-33 and -45 and HPV-58 and *HMBS*. RT-PCR reaction conditions and protocols have been described previously [28].

Each run was performed in 96-well plates, 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 viral load was normalised to cellular DNA input using a previously described formula (Equation 1) [15]. Absolute and normalised viral loads were both  $\log_{10}$  transformed.

Normalised viral load formula

$$\text{HPV DNA load (HPV copies/cell)} = \frac{\text{Number of HPV copies}}{(\text{Number of HMBS copies}/2)} \quad (1)$$

**Table 1 The probes and quenchers used for real-time polymerase chain reaction**

Test	Viral type	Size (bp)	Probe	Quencher
Reaction 1	HPV-16	78	FAM	ZEN/IBFQ
Reaction 2	HPV-18	80	Cy5	IBRQ
	HPV-31	78	HEX	ZEN/IBFQ
Reaction 3	HPV-33	78	FAM	ZEN/IBFQ
	HPV-45	76	Cy5	IBRQ
Reaction 4	HPV-58	109	HEX	ZEN/IBFQ
	<i>HMBS</i>	76	FAM	ZEN/IBFQ

Four parallel duplex real-time PCRs were performed per patient. Probe design for each viral type and *HMBS* was adjusted based on the types included in each reaction.

HPV: human papillomavirus; FAM: 6-carboxyfluorescein; Cy5: FluoroLink mono reactive dye Cy5; HEX: hexachlorofluoresceine; *HMBS*: hydroxymethylbilane synthase; ZEN/IBFQ: ZEN and Iowa Black FQ; IBRQ: Iowa Black RQ.

#### Statistical analysis

Sample size was calculated using the difference of proportions test for high viral load between women having and without cervical lesions (0.42 and 0.052 respectively) [8,30]; 0.05 significance, 90% statistical power and a 1:2 ratio between both groups were established. This meant that at least 23 women with lesions and 46 women without them were required for the study. Based on the availability of women without CIN, two women without cervical lesions reported by colposcopy were matched to each woman with CIN by age (within 5 years) and date of enrolment. As only a limited amount of women had

CIN 2+ or high-grade squamous intraepithelial lesions (CIN 2+, according to The Bethesda System (TBS)), CIN category was established which included women having CIN 2+ and women with CIN 1 or low-grade squamous intraepithelial lesions (CIN 1, according to TBS) [31,32] to improve the quality of the present study's statistical analysis.

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 [19].

Categorical variable differences between groups were assessed by Chi-squared test or Fisher's test, as appropriate, using a 0.05 significance level. Median and interquartile ranges (IQR) were used for quantitative variables, according to the data distribution.

HPV DNA load distribution between women according to colposcopy and biopsy results was analysed by the Mann–Whitney *U* test or Kruskal Wallis test, depending on the number of groups to be compared. Both absolute HPV DNA load and normalised HPV DNA load were analysed. Absolute viral load was categorised according to percentile distribution in both groups of patients as follows: negative  $\leq 0$ , low  $0 < VL \leq 10^5$  HPV copies and high  $>10^5$  HPV copies (to ensure better quality analysis).

Considering that women with CIN were paired with women without CIN by age and date of entering the study, conditional logistic regression was used for assessing the association between the HPV DNA load for each viral type and cervical lesion frequency according to colposcopy results. This analysis was not done taking the presence of biopsy-defined cervical lesions as outcome, as histology results were not available for all patients included in the study. Crude odds ratio (OR) and adjusted OR with their 95% confidence intervals (CI) were estimated, taking control variables into account, such as origin, ethnicity, age on starting to have sexual relations and the number of infecting HPV types. Hypothesis testing involved a two-tailed test (0.05 significance); STATA 10 was used for all statistical analysis.

## Results

180 patients fulfilled the inclusion criteria; 7 of them were excluded from statistical analysis, as their *HMBS* gene could not be amplified. This meant that 114 women were classified as negative for intraepithelial lesions (92.98% having normal cytology) and 59 women having CIN identified by colposcopy (56 women having CIN 1 and 3 having CIN 2+) were included in the analysis (Figure 1).

According to the diagnostic algorithm, a biopsy was taken from 59 women having colposcopy-defined cervical lesions; however, results were only obtained for 45 women as the samples taken for pathology regarding the

remaining 14 women were unsatisfactory or had been lost. 23.73% ( $n = 14$ ) of the women had confirmation of CIN 1 by biopsy (only one woman with CIN 2+ was found). Two of the CIN 2+ women detected by colposcopy had CIN 1 by biopsy.

Regarding women with CIN, median age was 40 years old (14 years IQR) and 41.5 years old (13 years IQR) in women without CIN. Most women participating in the study came from the city of Girardot (60.69%;  $n = 105$ ); 76.19% ( $n = 80$ ) of these women were negative for lesions. 95.95% of the women in the study were mestizos ( $n = 166$ ) and the remaining percentage (4.05%) was made up of indigenous, white and black women. The distribution of socio-demographic characteristics and risk factors associated with CC and the detection of HPV infection was compared between both groups (those with CIN and those without it), significant differences being found regarding origin ( $p < 0.05$ ) (Table 2).

Overall, 91.91% ( $n = 159$ ) of the sample proved positive for the detection of HPV by RT-PCR, i.e. 93.22% ( $n = 55$ ) of women with CIN (92.86% positive from the group having CIN 1 and 100% positive from the group having CIN 2+) and 91.23% ( $n = 104$ ) of women without lesions. 79.24% ( $n = 126$ ) of all infected women were infected by more than one viral type; this was observed in 81.82% ( $n = 45$ ) of women with CIN and 77.88% ( $n = 81$ ) of women negative for lesions. Simultaneous infection was more frequent concerning 2 high-risk viral types in women without lesions ( $n = 29$ ; 27.88%) and 3 types in women with cervical lesions ( $n = 19$ ; 34.54%). The most frequently encountered viral types were HPV-18 and HPV-16 in multiple infections, in both groups.

The type-specific distribution revealed HPV-18 as being most frequent in both groups (69.49% in women having CIN and 66.66% in women without CIN), followed by HPV-16 (57.63%) and HPV-45 (38.98%) in women having lesions and HPV-16 (45.61%), HPV-31 (45.61%) and HPV-45 (38.60%) in women proving negative for lesions. HPV-33 had the lowest infection frequency in both groups.

Higher high viral load was recorded concerning HPV-18, HPV-16 and HPV-33 infection in women with CIN, whilst high viral load was most frequent in HPV-31, HPV-45 and HPV-58 infection in women without lesions (Table 3).

Figures 2 shows absolute (A) and normalised (B) viral load distribution for each HR-HPV type, comparing both groups of women. It is worth stating that HPV-31 (in women without CIN) and HPV-33 (in women having CIN) were the HR-HPV viral types having the highest absolute viral load (median = 9.41 (2.58 IQR) HPV copies for HPV-31 and median = 9.33 (2.94 IQR) HPV copies for HPV-33) whilst HPV-58 infection had the lowest absolute viral load in both groups of women. The

**Table 2 The distribution of socio-demographic characteristics and risk factors**

Characteristic	Categories	n	%	With CIN (n = 59)		Without CIN (n =114)		p
				n	%	n	%	
Age, years	<30	29	16.76	11	18.64	18	15.79	0.493
	30–40	54	31.21	21	35.59	33	28.95	
	>40	90	52.02	27	45.76	63	55.26	
Origin	Bogotá	65	37.57	32	54.24	33	28.95	<b>0.001</b>
	Other city	108	62.43	27	45.76	81	71.05	
Ethnicity	Other	7	4.05	3	5.08	4	3.51	0.691
	Mestizo	166	95.95	56	94.92	110	96.49	
Average monthly income*	≤ minimum	155	89.06	53	89.83	102	89.47	0.942
	>minimum	18	10.40	6	10.17	12	10.53	
Educational level	No schooling	1	0.58	1	1.69	0	0.00	0.094
	Primary	82	47.40	22	37.29	60	52.63	
	Secondary	74	42.77	28	47.46	46	40.35	
	Technical	10	5.78	6	10.17	4	3.51	
	Graduate	6	3.47	2	3.39	4	3.51	
Marital status	Single	17	9.83	4	6.78	13	11.40	0.673
	Married	20	11.56	7	11.86	13	11.40	
	Divorced	8	4.62	4	6.78	4	3.51	
	Living with partner	126	72.83	43	72.88	83	72.81	
	Widow	2	1.16	1	1.69	1	0.88	
Healthcare scheme affiliation	Subsidised- linked	159	91.91	52	88.14	107	93.86	0.191
	Contributory-private	14	8.09	7	11.86	7	6.14	
Smoker	No	146	84.39	49	83.05	97	85.09	0.726
	Yes	27	15.61	10	16.95	17	14.91	
Age at first intercourse, years	<16	41	23.70	10	16.95	31	27.19	0.133
	≥16	132	76.30	49	83.05	83	72.81	
Lifetime number of sexual partners	1	72	41.62	26	44.07	46	40.35	0.868
	2–3	84	48.55	27	45.76	57	50.00	
	>3	17	9.83	6	10.17	11	9.65	
Contraceptive method	None	65	37.57	19	32.20	46	40.35	0.697
	Surgery	52	30.06	15	25.42	22	19.30	
	Hormonal	19	10.98	18	30.51	34	29.82	
	Barrier	37	21.39	7	11.86	12	10.53	
Pregnancies	None	4	2.31	1	1.69	3	2.63	0.326
	1–2	76	43.93	28	47.46	48	42.11	
	3–4	74	42.77	27	45.76	47	41.23	
	>4	19	10.98	3	5.08	16	14.04	
Abortions	None	82	47.40	27	45.76	55	48.25	0.818
	1	68	39.31	25	42.37	43	37.72	
	≥2	23	13.29	7	11.86	16	14.04	
STD	No	137	79.19	47	79.66	90	78.95	0.913
	Yes	36	20.81	12	20.34	24	21.05	

Values in bold =  $p < 0.05$ .

\*The minimum average monthly income (2014 rate) would be roughly US \$300.

p = p value; CIN: cervical intraepithelial neoplasia; STD: sexually transmitted disease.

**Table 3 Type-specific HR-HPV viral load distribution by category**

HPV type	n	%	With CIN (n = 59)						Without CIN (n = 114)						p
			Negative		Low viral load		High viral load		Negative		Low viral load		High viral load		
			n	%	n	%	n	%	n	%	n	%	n	%	
HPV-16	86	49.71	25	42.37	12	20.34	22	37.29	62	54.39	13	11.40	39	34.21	0.186
HPV-18	117	67.63	18	30.51	10	16.95	31	52.54	38	33.33	18	15.79	58	50.88	0.928
HPV-31	71	41.04	40	67.80	1	1.69	18	30.51	62	54.39	3	2.63	49	42.98	0.257
HPV-33	14	8.09	54	91.53	0	0.00	5	8.47	105	92.11	1	0.88	8	7.02	0.846
HPV-45	67	38.73	36	61.02	9	15.25	14	23.73	70	61.40	10	8.77	34	29.82	0.366
HPV-58	56	32.37	42	71.19	7	11.86	10	16.95	75	65.79	16	14.04	23	20.18	0.772
HR-HPV*	159	91.91	4	6.78	8	13.56	47	79.66	10	8.77	12	10.53	92	80.70	0.777

HPV DNA load: categorised as  $\leq 0$  = negative,  $0 < VL \leq 10^5$  HPV copies = low viral load,  $> 10^5$  HPV copies = high viral load.

\*HR-HPV: high risk-human papillomavirus, infection by at least one high-risk viral type from the 6 analysed here.

HPV: human papillomavirus; CIN: cervical intraepithelial neoplasia; p = p value.

range of values for normalised viral load was lower than for absolute (up to  $10^8$  HPV copies). The highest absolute viral load was detected for HPV-31 in women with CIN ( $10^{22}$  HPV copies) and highest normalised viral load for HPV-33 in women without CIN. No statistically significant differences were observed regarding viral load distribution (absolute and normalised) for each HR-HPV type in either group of patients.

The three patients having CIN 2+ were positive for HR-HPV; HPV-18 and HPV-31 were detected in two of them, whilst the other one was positive for HPV-18, HPV-16 and HPV-45. Even though women having CIN 2+ had a higher viral load (normalised for HPV-18 and absolute for HPV-16) than women having CIN 1, the differences in viral load distribution were not statistically significant. However, normalised viral load for HPV-31 was greater in women negative for cervical lesion and having CIN 1 compared to women having CIN 2+ (marginal significance, i.e.  $p = 0.052$ ).

The distribution of viral load was also analysed for each HR-HPV type, according to biopsy result. Similar results were found to those with colposcopy (i.e. higher absolute viral loads in women having a severer degree of lesion); and for some types (HPV-31, HPV-33 and HPV-58) higher normalised viral loads; however, the differences were not statistically significant due to the amount of women analysed (Table 4).

Crude and adjusted odds ratios (OR) were calculated for estimating the magnitude of absolute viral load association with CIN for each viral type. The conditional logistic regression model revealed that HPV-16 infection was significantly associated with greater frequency regarding cervical lesions. However, lesions occurred more frequently in the group of women having low viral load for HPV-16 ( $0 < VL \leq 5.86$  HPV copies) than in women having a high load ( $> 5.86$  HPV copies), (3.53  $OR_{adj}$ , 1.16–10.74 95%CI; 2.63  $OR_{adj}$ , 1.09–6.36 95%CI, respectively). It was also found that CIN frequency was lower in women having HPV-31 and

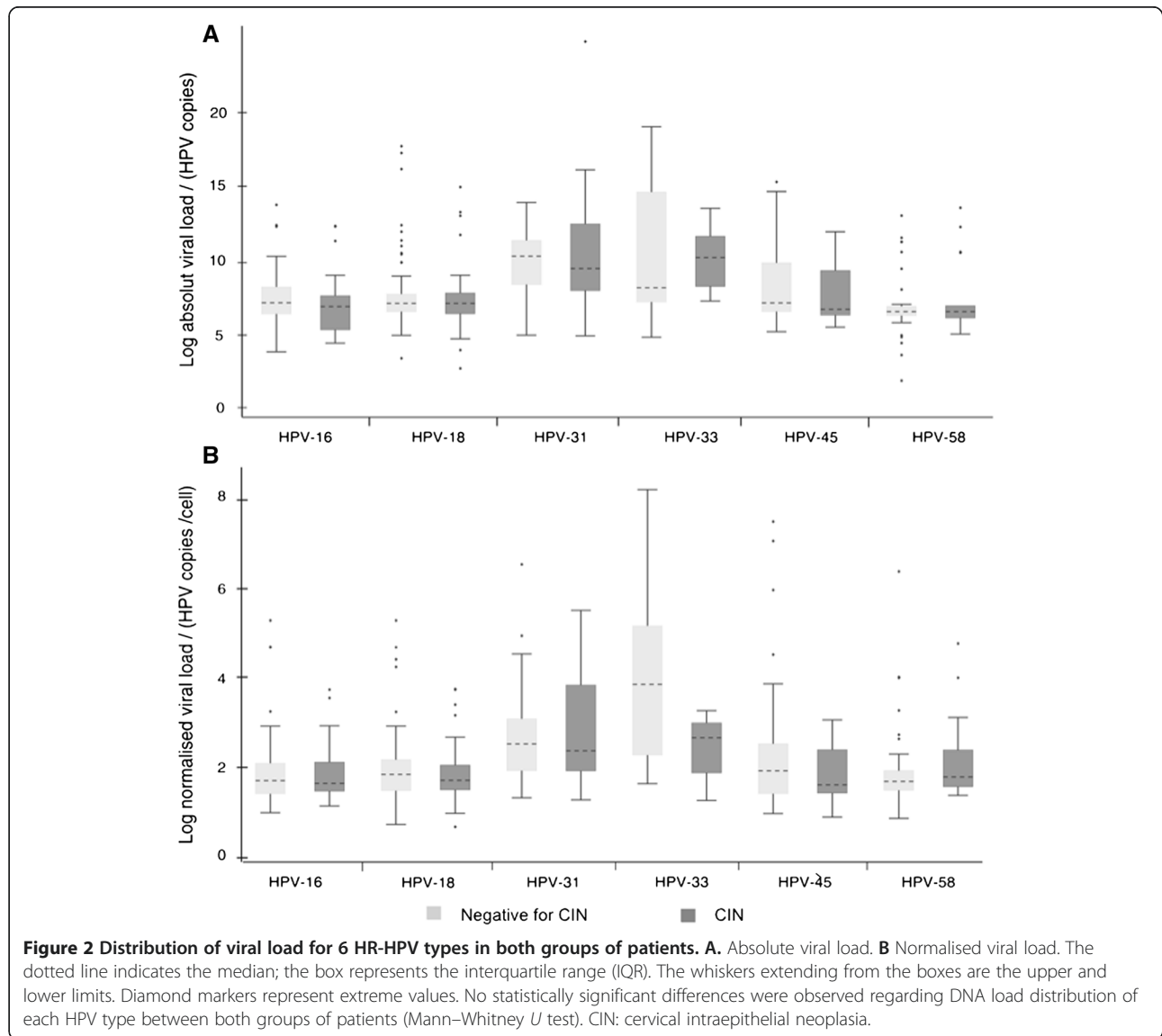
high viral load ( $> 5.14$  HPV copies; 0.34  $OR_{adj}$ , 0.15–0.78 95%CI). No significant associations were obtained for the other viral types with the presence of CIN (Table 5).

## Discussion

This study involved using RT-PCR; this enabled type-specific evaluation of the viral load of the most frequently occurring oncogenic types in Colombia (HPV-16, -18, -31, -33, -45 and -58) [19] for determining each type's association with precursor lesions of CC. As the method has high sensitivity, specificity and has a broad dynamic range of viral detection (up to  $10^{22}$  HPV copies) this provided the best approach for this study [12,13,16,18,33].

More HPV infections were found in women having CIN in our sample, amongst whom all women having CIN 2+ were HPV positive. The foregoing was consistent with the fact that almost 99.7% of CC cases are associated with HPV [1]. Previous studies have demonstrated that HPV prevalence in women having CIN is high, proportionally increasing as lesion severity increases [30,34,35]. The prevalence found here was greater than that reported in the literature (100% in CIN 2+, 92.86% in CIN 1 and 91.23% in women without CIN). Women were included in this study who had been previously identified as HPV positive using conventional PCR; this explained the high prevalence of HPV when using RT-PCR in women without lesions. However, variable infection prevalence in women without CIN has been found worldwide (mean = 12.6%) [35,36].

Multiple infection frequency has been variable (16.3%–55%) in previous reports concerning women having lesions [35]; up to 3.4% infection by multiple types of HR-HPV has been described in women without lesions [37]. The present study revealed more multiple infections (in both the general population and women having CIN and those without them) regarding previous reports worldwide, but similar to that previously reported in Colombia [27,38]. However, RT-PCR was used which has high



**Table 4 Distribution of 6 HR-HPV types’ viral load regarding biopsy results**

Viral type	Negative (n = 28)		CIN 1 (n = 16)			CIN 2+ (n = 1)			
	% (n)	Viral load, median (IQR)		% (n)	Viral load, median (IQR)		% (n)	Viral load, median (IQR)	
		Absolute	Normalised*		Absolute	Normalised*		Absolute	Normalised*
HPV-16	66.67 (22)	6.42 (1.69)	1.79 (0.54)	57.89 (11)	6.77 (3.04)	1.69 (0.64)	0	n/a	n/a
HPV-18	66.67 (22)	6.29 (1.34)	1.84 (0.51)	68.42 (13)	6.61 (2.28)	1.67 (1.79)	100 (1)	7.02 (n/a)	2.07 (n/a)
HPV-31	30.30 (10)	8.51 (1.90)	2.39 (0.38)	31.58 (6)	9.69 (6.00)	3.50 (2.17)	0	n/a	n/a
HPV-33	3.03 (1)	6.75 (n/a)	1.98 (n/a)	10.53 (2)	8.48 (1.70)	2.37 (2.06)	100 (1)	10.57 (n/a)	3.13 (n/a)
HPV-45	51.52 (17)	6.13 (2.95)	1.79 (1.00)	42.11 (8)	6.24 (1.17)	1.61 (0.80)	0	n/a	n/a
HPV-58	21.21 (7)	5.93 (3.89)	2.14 (2.35)	36.84 (7)	6.12 (0.34)	1.75 (0.28)	0	n/a	n/a
HR-HPV**	94.34 (31)	6.37 (1.20)	2.06 (0.63)	94.74 (18)	6.77 (2.97)	2.12 (1.37)	100 (1)	8.80 (n/a)	2.60 (n/a)

Absolute and normalised viral loads were both log<sub>10</sub> transformed.

\*HPV copies/cell = number of HPV copies/(number of HMBS copies/2).

\*\*HR-HPV: high risk-human papillomavirus, infection by at least one high-risk viral type from the 6 analysed here.

HPV: human papillomavirus; CIN: cervical intraepithelial neoplasia; CIN 1: cervical intraepithelial neoplasia 1; CIN 2+: cervical intraepithelial neoplasia 2 or 3; n/a: not applicable.

**Table 5 Conditional logistic regression model**

HPV type	Viral load	With CIN / without CIN	Crude OR (95%CI)	Adjusted OR*	95%CI
HPV-16	Negative	25/62	Reference		
	<b>0 &lt; VL ≤ 5.86</b>	12/13	2.19 (0.88–5.43)	<b>3.53</b>	<b>1.16–10.74</b>
	<b>5.86 &lt; VL</b>	22/39	1.27 (0.64–2.50)	<b>2.63</b>	<b>1.09–6.36</b>
HPV-18	Negative	18/38	Reference		
	<b>0 &lt; VL ≤ 5.95</b>	10/18	1.14 (0.45–2.89)	1.72	0.52–5.69
	<b>5.95 &lt; VL</b>	31/58	1.06 (0.52–2.17)	1.77	0.68–4.63
HPV-31	Negative	40/62	Reference		
	<b>0 &lt; VL ≤ 5.14</b>	1/3	0.52 (0.04–6.29)	0.15	0.01–2.26
	<b>5.14 &lt; VL</b>	18/49	0.60 (0.32–1.14)	<b>0.34</b>	<b>0.15–0.78</b>
HPV-33	Negative	54/105	Reference		
	<b>0 &lt; VL ≤ 4.60</b>	0/1	0.00 (0 - .)	0	0 - .
	<b>4.60 &lt; VL</b>	5/8	1.43 (0.45–4.50)	1.67	0.44–6.28
HPV-45	Negative	36/70	Reference		
	<b>0 &lt; VL ≤ 5.98</b>	9/10	1.53 (0.60–3.92)	2.94	0.92–9.44
	<b>5.98 &lt; VL</b>	14/34	0.79 (0.38–1.67)	1.13	0.43–2.96
HPV-58	Negative	42/75	Reference		
	<b>0 &lt; VL ≤ 5.97</b>	7/16	0.83 (0.32–2.11)	0.73	0.23–2.31
	<b>5.97 &lt; VL</b>	10/23	0.83 (0.37–1.83)	0.86	0.35–2.12
HR-HPV**	Negative	4/10	Reference		
	<b>0 &lt; VL ≤ 5.94</b>	8/12	1.73 (0.40–7.47)	1.01	0.23–4.50
	<b>5.94 &lt; VL</b>	47/92	1.18 (0.35–4.00)	1.39	0.25–7.81

Values in bold =  $p < 0.05$ .

\*Adjusted for origin, ethnicity, age at first intercourse and number of viral types.

\*\*HR-HPV: high-risk-human papillomavirus, infection by at least one high-risk viral type from the 6 analysed here (viral load = sum of viral loads of HPV types detected/ number of HPV types detected).

HPV: human papillomavirus; CIN: cervical intraepithelial neoplasia; VL: viral load; OR: odds ratio.

sensitivity and allows small amounts of viral DNA to be detected, compared to other methods [13,18]. This has been previously demonstrated by studies carried out involving RT-PCR which have reported high multiple infection frequency [39,40]. Such differences regarding co-infection prevalence reported in various studies might have been due to their design, sample size, the HPV detection methods used and the population being studied (geographic, demographic and clinical factors) [37].

HPV-18 and HPV-16 occurred most frequently in the present study, followed by HPV-45 and HPV-58. Differences concerning type-specific prevalence have been reported according to geographic and demographic factors [3,35]. It is worth noting that the two most common types found here are responsible for the 70% of cases of CC [41] and that the HPV genotypes evaluated in this study have been reported amongst the 8 HR-HPV types most frequently occurring around the world, in both women without lesions and women with CC [2,3,35].

Absolute viral load was highest in women having CIN compared to women without lesions determined by both colposcopy and biopsy; an increase in the viral load was observed for HPV-18 and HPV-33 proportional to the

degree of injury. The foregoing was consistent with previous studies which have revealed the effect of viral load on developing CC. Most HPV-16 studies have found that viral load has increased in relation to the degree of cervical lesion severity [8-11,15,16,42].

An association between viral load and cervical lesion frequency (as assessed by colposcopy) was observed in this study just for HPV-16 and HPV-31. The present study's results highlighted the fact that women having low HPV-16 load (<5.86 HPV copies) had higher cervical lesion frequency. Such results agreed with those from a study by Manawapat, Stubenrauch *et al.*, [43] which showed that women having persistent HPV-16 infection had lower viral load than those who had a transient infection (4.72 copies/cell *cf* 20 copies/cell;  $p = 0.0003$ ). It has been found recently that low viral load was characteristic of intermittently detected persistent infection [44]. Reduced viral load has been described in women having CIN; this has been explained by HPV genome integration associated with down-regulation of viral DNA synthesis, thereby affecting immune system activation and thus reducing the probability of infection being eliminated [43,45-47]. Accordingly, a long period of

latency accompanied by low viral load would probably be observed, representing a greater risk for infection persistence and lesion progression [48].

Contrary to our findings regarding HPV-16 viral load, the present study found that a high HPV-31 load (>5.14 HPV copies) was associated with lower cervical lesion frequency. As mentioned previously regarding HPV-16 results, it has been shown that viral load has been greater in transitory infections regarding patients having persistent infection [43]. This agreed with the finding that clearance of HPV-16 infection has been preceded by a transient viral load peak or a plateau phase [33]; such high load was probably necessary for the immunological system to become induced, thereby favouring HPV elimination. According to the above, HPV-31 infections are probably transitory and such association is mediated by an immune system response to high viral load which can eliminate the infection and thus CC precursor lesions do not progress or such lesions regress spontaneously [47].

Regarding the other viral types (HPV-18, -33, -45 and -58), no association was found between viral load and cervical lesion frequency; such result was supported by data from other authors [14-16,42,49,50]. However, a study by Moberg, Gustavsson *et al.*, found that high HPV-16, HPV-31 and HPV-18/45 viral load increased the risk of developing carcinoma *in situ* (CIS) [51].

The pertinent literature gives different cut-off points when categorising viral load, depending on the quantification technique used (RT-PCR, Hybrid Capture II (HCII)) [8] and distribution in a particular population being evaluated [9,51]. A study which evaluated the clinical significance of HPV-16 and -18 viral loads determined that HPV-16 viral load was related to cervical lesion severity, having a  $3.0 \times 10^6$  copies/million cells threshold, this being highly specific for grade 2 diagnosis [15]. Taking the foregoing into account, viral load was categorised in the present study according to percentile distribution, leaving  $10^6$  copies as cut-off point for ensuring analysis quality.

It is worth stressing that this technique managed to detect a broad range of viral load, even after stratifying by colposcopy result and viral type. However, this hampered establishing viral load cut-off points to enable identifying women at greater risk of developing cervical lesions; previous studies have also experienced such difficulty [12,16,33].

This work's value lies in it being a study where a reproducible, sensitive and specific technique (i.e. RT-PCR) was used for detecting and quantifying viral load (absolute and normalised) not just for one viral type but for the 6 most frequently occurring high-risk HPV types described to date in Colombia. Besides, this is the first study carried out in Colombia which has included

women from regions having high HPV infection prevalence and which was aimed at evaluating the association between HPV viral load and cervical lesion frequency.

This study's results were obtained from a single evaluation of HPV viral load; this means that predicting the risk of lesion progression and developing CC later on cannot be ascertained from this. However, it can be stated that our results were consistent with some findings reported in longitudinal studies [33,43,44,48]. The infection duration time of the women included in this study was also unknown; HPV-16 might thus have been greater in women having CIN and lower in HPV-31 women. Another limitation of this study was the low number of women having CIN 2+ which hindered generalising the results to all CC precursor lesions. An analysis of HPV viral load dynamics could thus be more reliable and provide more information for estimating whether HPV infection will worsen or clear and predicting the development of CC or cervical lesions. Prospective studies on women having HPV infection which would include type-specific determination (according to local prevalence) of viral load and women having cervical lesions with different degrees of severity are thus needed for confirming our results.

## Conclusions

A significant association was found in this study, low HPV-16 and high HPV-31 viral loads were associated with higher CIN frequency; this might have been related to infection duration and immune system response. HPV infection's effect on developing CC is influenced by viral load, meaning that measuring load could improve the predictive value of HPV detection; however, the scope of quantification depends on the viral type being detected. These findings support the idea of quantifying viral load (as a type-specific marker of CC), coupled to cytology, for improving and strengthening CC screening programmes. This would lead to identifying HPV positive women at greater risk of developing cervical lesions, as well as identifying women as yet lacking cervical anomalies for predicting the beginnings of neoplasia.

## Abbreviations

HPV: Human papillomavirus; HR-HPV: High-risk human papillomavirus; CC: Cervical cancer; CIN: Cervical intraepithelial neoplasia; CIN 1: Cervical intraepithelial neoplasia 1; CIN 2+: Cervical intraepithelial neoplasia 2 or 3; HMBS: Hydroxymethylbilane synthase; PCR: Polymerase chain reaction; RT-PCR: Real-time polymerase chain reaction; DNA: Deoxyribonucleic acid; VL: Viral load; STD: Sexually-transmitted diseases; HC II: Hybrid capture II; FAM: 6-carboxyfluorescein; Cy5: FluoroLink mono reactive dye Cy5; HEX: hexachlorofluorescein; ZEN/IBFQ: ZEN and IOWA Black FQ; IBRO: IOWA Black RQ; SD: Standard deviation; CI: Confidence interval; IQR: Interquartile range; n/a: Not applicable; OR: Odds ratio.

## Competing interests

All authors declare that they have no competing interests.

### Authors' contributions

All the authors were involved in developing the study and preparing the ensuing article. LDRO and SCSDL provided the concept and designed the study, as well as acquiring, analysing and interpreting the data and writing the article. MC helped draft the manuscript and assisted with data analysis. DAMP developed the methodology and was 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 revised the document and lent their expertise regarding the discussion of results. All authors have read and approved the final version of the manuscript.

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### Author details

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

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RESEARCH ARTICLE

# *Chlamydia trachomatis* Frequency in a Cohort of HPV-Infected Colombian Women

Edith Margarita Quinónez-Calvache<sup>1,2</sup>✉, Dora Inés Ríos-Chaparro<sup>3</sup>✉, Juan David Ramírez<sup>3</sup>, Sara Cecilia Soto-De León<sup>1,4</sup>, Milena Camargo<sup>1,4</sup>, Luisa Del Río-Ospina<sup>1,5</sup>, Ricardo Sánchez<sup>1,5</sup>, Manuel Elkin Patarroyo<sup>1,5</sup>, Manuel Alfonso Patarroyo<sup>1,4\*</sup>

**1** Molecular Biology and Immunology Department, Fundación Instituto de Inmunología de Colombia (FIDIC), Bogotá, Colombia, **2** Biotechnology Institute, Faculty of Sciences, Universidad Nacional de Colombia, Bogotá, Colombia, **3** Grupo de Investigaciones Microbiológicas–UR (GIMUR), Faculty of Natural and Mathematical Sciences, Universidad del Rosario, Bogotá, Colombia, **4** School of Medicine and Health Sciences, Universidad del Rosario, Bogotá, Colombia, **5** School of Medicine, Universidad Nacional de Colombia, Bogotá, Colombia

✉ These authors contributed equally to this work.

\* [mapatarr.fidic@gmail.com](mailto:mapatarr.fidic@gmail.com)



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**Abbreviations:** *C. trachomatis*, *Chlamydia trachomatis*; HPV, human papillomavirus; HR-HPV, high-risk human papillomavirus; CC, cervical cancer; CIN, cervical intraepithelial neoplasia; PCR, polymerase chain reaction; DNA, deoxyribonucleic

## Abstract

### Background

*Chlamydia trachomatis* (*C. trachomatis*), an obligate intracellular bacterium, is the commonest infectious bacterial agent of sexual transmission throughout the world. It has been shown that the presence of this bacteria in the cervix represents a risk regarding HPV persistence and, thereafter, in developing cervical cancer (CC). Prevalence rates may vary from 2% to 17% in asymptomatic females, depending on the population being analysed. This study reports the identification of *C. trachomatis* in a cohort of 219 HPV-infected Colombian females.

### Methods

*C. trachomatis* infection frequency was determined during each of the study's follow-up visits; it was detected by amplifying the cryptic plasmid sequence by polymerase chain reaction (PCR) using two sets of primers: KL5/KL6 and KL1/KL2.

Infection was defined as a positive PCR result using either set of primers at any time during the study. Cox proportional risk models were used for evaluating the association between the appearance of infection and a group of independent variables.

### Results

Base line *C. trachomatis* infection frequency was 28% (n = 61). Most females infected by *C. trachomatis* were infected by multiple types of HPV (77.42%), greater prevalence occurring in females infected with HPV-16 (19.18%), followed by HPV-58 (17.81%). It was observed that females having had the most sexual partners (HR = 6.44: 1.59–26.05 95%CI) or infection with multiple types of HPV (HR = 2.85: 1.22–6.63 95%CI) had the greatest risk of developing *C. trachomatis*.

acid; STI, sexually-transmitted infection; SD, standard deviation; CI, confidence interval; HR, hazard ratio; ASC-H, atypical squamous cells not exclude H-SIL; ASC-US, atypical squamous cells of undetermined significance; L-SIL, low squamous intraepithelial lesion; H-SIL, high squamous intraepithelial lesion.

## Conclusions

The study provides data regarding the epidemiology of *C. trachomatis* /HPV coinfection in different population groups of Colombian females and contributes towards understanding the natural history of *C. trachomatis* infection.

## Introduction

*Chlamydia trachomatis* (*C. trachomatis*) is an obligate intracellular bacterium which can infect both ocular and genital tissues. Infection by *C. trachomatis* causes chronic inflammation damaged epithelial tissue and pelvic inflammatory disease (PID). Due to being obligate and intracellular, the pathogen persists in a host after an immune response has been developed, meaning that it could produce chronic disease, causing damage to tissue, resistance to apoptosis and affecting host ability to clear other STI infections [1, 2].

*C. trachomatis* is the commonest infectious bacterial agent of sexual transmission throughout the world [3, 4]. The WHO has estimated that about 98 million adults were infected by *C. trachomatis* in 2005 and 101 million new cases of *C. trachomatis* occur annually around the world [4, 5]. Prevalence rates may vary from 2% to 17% in asymptomatic females, depending on the population and country evaluated [6]. *C. trachomatis* prevalence has been reported as being 1.4% for males and 1.6% for females in people aged 18–44 years old, the highest rates occurring in subjects aged 18 to 29 years old: 2.5% for males and 3.2% for females. Its prevalence in sexually-transmitted infection (STI) detection centres has been seen to rise to 15% [7].

*C. trachomatis* infection prevalence and determinants in Colombia have not been described completely. Some data have been reported by the Colombian Ministry of Health and Social Protection indicating that 1,538, 1,525 and 1,313 people were diagnosed as being infected by *C. trachomatis* in 2009, 2010 and 2011, respectively. It has also been indicated that this is the most prevalent STI in people aged 15 to 49 years-old [8].

The asymptomatic nature of *C. trachomatis* infection, its long-term persistence and its ability to induce chronic inflammation and metaplasia has led to this bacteria being considered a potential cofactor in high-risk (HR) human papilloma virus (HPV) infection since it could promote the virus' persistence and/or potentiate its oncogenicity [2]. Besides inducing tissue inflammation, it also affects host ability to purge HPV infection, thereby contributing towards viral persistence and increasing the risk of developing lesions having a poor prognosis [9].

HR-HPV types are considered the main aetiological agents of cervical neoplasia (CN) [10], however, only a small percentage of HPV-infected females progress to invasive cervical cancer (ICC), meaning that developing it has been associated with other cofactors acting together with HPV [11], such as host-related (e.g. endogenous hormones, genetic background and immune response) and virus-related ones (e.g. load, viral integration and concomitant infection by other STI agents such as HIV and *C. trachomatis*) [12].

The importance of such infection in terms of public health means that HPV and *C. trachomatis* coinfection frequency must be estimated regarding females with/without cervical lesions [10] associated with possible reinfection and final outcome, bearing in mind that identifying *C. trachomatis* when there is coinfection with HPV has been poorly evaluated in Colombia to date [13]. This study was thus aimed at determining *C. trachomatis* frequency in a population of HPV-infected females living in 3 cities in Colombia (Bogotá, Chaparral and Girardot). This is the first study which has evaluated a cohort which was completely infected with HPV at the

start; the aim was to determine *C. trachomatis* infection incidence and the risk factors associated with HPV coinfection.

## Materials and Methods

### Study population

The population studied consisted of 219 females whose ages ranged from 17–71 years old. A previous study has described how the cohort was assembled for identifying HPV, involving 3 follow-ups every 6 months ( $\pm 3$  months) [14].

The females in the study were living in 3 cities in Colombia: Bogotá, the capital city, Chaparral in the Tolima department and Girardot in the Cundinamarca department, where intrinsic characteristics have been identified in previous studies regarding the risk of contracting HPV infection [15]. Chaparral and Girardot were grouped under the “other city” category to facilitate statistical analysis.

### Ethics, consent and permissions

The females involved in the study carried out by the Fundación Instituto de Inmunología de Colombia (FIDIC) voluntarily decided to participate in sampling between April 2007 and March 2010; they signed an informed consent form which explained the risk factors associated with the exams and sample taking. A parent or guardian/teacher had to sign for girls aged less than 18 years old. All the procedures were approved by the following hospitals’ ethics’ committees: Hospital de Engativá (Bogotá population), Hospital San Juan Bautista (Chaparral) and Nuevo Hospital San Rafael (Girardot). *C. trachomatis* was determined from samples taken from the 219 HPV-infected females at the start of the follow-up and thereafter during each visit whilst the study lasted.

### Obtaining biological material

Biological material was obtained from cervical smear samples collected after cytology and stored at  $-20^{\circ}\text{C}$ . The samples were processed and DNA extracted, according to information previously published by our group [14].

### Molecular detection of *C. trachomatis*

Polymerase chain reaction (PCR) amplification of a sequence from the microorganism’s cryptic plasmid was used for detecting *C. trachomatis* in samples from HPV-infected females. Two sets of primers previously reported in the literature were used; KL5/KL6 [16, 17] amplified a 350 bp conserved cryptic multicopy plasmid fragment and KL1/KL2 a 241 bp fragment [2, 18, 19] (Table 1). The reactions with each set of primers were done separately and simultaneously for each sample.

Amplification conditions were chosen and adjusted according to the methodologies described for *C. trachomatis* detection [2, 10, 18, 19]. The *C. trachomatis* ATCC UW-36/Cx

**Table 1. Primers used for amplifying *Chlamydia trachomatis*.**

Region	Name of primer	Sequence
pLGV440	KL5	5'- TTT GCC TTA ACC CCA CCA TT-3'
	KL6	5'- CGT CCT TCC TAA AAG AGC TA -3'
pLGV440	KL1	5'- TCC GGA GCG AGT TAC TAA GA -3'
	KL2	5'- AAT CAA TGC CCG GGA TTG GT -3'

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strain (ATCC VR-886D) was used as positive control for each amplification and DNase-free water as reagent control. The PCR for each set of primers was done at 20  $\mu$ L final volume, using 3  $\mu$ L of sample, with approximately 900 ng of DNA.

Reaction conditions for KL5 and KL6 were: 1X concentration Bioline buffer (KCl, Tris HCl, pH = 8.3), 3mM MgCl<sub>2</sub>, 1.25mM each desoxynucleotide triphosphate (dNTP), 20 pmol of each primer and 1.25  $\mu$ L of Taq DNA polymerase (5U/ $\mu$ L). This set of primers' thermal profile involved an initial cycle at 94°C for 5 minutes, followed by 35 cycles of 1 minute at 93°C, 1 min at 56.8°C, 1 min at 72°C and a final extension step at 72°C for 10 minutes.

Reaction conditions for KL1 and KL2 were: Bioline buffer (KCl, Tris HCl pH = 8.3) at 1X concentration, 3mM MgCl<sub>2</sub>, 1.25mM each desoxynucleotide triphosphate (dNTP), 20 pmol of each primer and 1.25  $\mu$ L of Taq DNA polymerase (5U/ $\mu$ L) (Bioline). This set of primers' thermal profile involved an initial cycle at 94°C for 5 minutes, followed by 35 cycles of 1 minute at 93°C, 1 min at 55°C, 1 min at 72°C and a final extension step at 72°C for 10 minutes.

Bovine serum albumin (BSA) was added to the molecular biology grade water used in the PCR reactions at 0.8  $\mu$ g/ $\mu$ L concentration; this was added to increase PCR efficiency as described earlier [20].

## Statistical analysis

*C. trachomatis* infection frequency was determined during each follow-up visit of the study. Means and their corresponding measures of dispersion (standard deviation) were used for describing continuous variables. Categorical variables were expressed in terms of frequencies and percentages.

The association between the presence of infection and a group of categorical variables was evaluated; these were: smoking, city, ethnicity, age on first sexual relationship, number of sexual partners, family planning method, presence of sexually-transmitted infection (STI), number of children, history of abortions, coinfection with HR-HPV, HPV16, HPV18, HPV31, HPV45, HPV33, HPV58, the amount of HPV infecting types, cytology (negative, ASC-H, ASC-US, LIE-BG, LIE-AG), colposcopy (negative, LIE-BG, LIE-AG, suggestive, unsatisfactory), using Chi<sup>2</sup> ( $\chi^2$ ) and Fisher's exact tests, depending on contingency table characteristics.

*C. trachomatis* infection was defined as a positive PCR result using either set of primers at any time during the study. Clearance was defined as *C. trachomatis* not being detected by both sets of primers following a positive finding during the previous visit. Persistence was defined as the presence of infection in two consecutive visits. Coinfection was defined as the simultaneous presence of *C. trachomatis* and HPV infection whilst the coexistence of two or more types of HPV was defined as multiple infection; such definitions were used according to that established in previous studies [21–23].

Two subpopulations were defined for the cohort study; the first involved patients who were not infected by *C. trachomatis* when the cohort study began (to evaluate infection incidence) whilst the second subpopulation involved females who were infected from the start (for evaluating clearance). Follow-up times were used for estimating Kaplan-Meier survival functions for each outcome (infection or clearance).

Infection (for those who started without *C. trachomatis*) and clearance rates (for those who were infected at the beginning) were estimated according to the information obtained. Time was calculated in terms of months elapsed since the beginning of follow-up until the event in question (infection or clearance). Cases where no event occurred and follow-up had ended were handled as right censoring for survival analysis.

Cox proportional risk models were used for evaluating the association between the appearance of infection and a group of independent variables, such as race, smoking, age on first

sexual relationship, family planning method, number of sexual partners, age, number of children, the presence of sexually-transmitted infection (STI) and coinfection with HR-HPV. The beginning of risk was assumed to be when the cohort began without infection and with follow-up until the presence of an infection event, or the end of the study considered a closed case. The strength of association between the independent variables and the risk of having infection was quantified by using a hazard ratio (HR) estimator, i.e. the ratio of hazard rates for the independent variables' different levels.

The hypothesis tests involved using 5% significance ( $p \leq 0.05$ ), 95% confidence interval (CI) for the estimator and two-tail hypothesis. STATA 12 software was used for all statistical analysis.

## Results

### The cohort's characteristics

Two hundred and nineteen cervical samples from females who started the cohort infected by HR-HPV (HPV-16, -18, -31, -33, -45 and -58) were analysed; these females were followed-up every 6 ( $\pm 3$ ) months. All the females made a minimum of 3 visits ( $n = 216$ ); however, some of them ( $n = 49$ ) made 5 visits. A positive or negative *C. trachomatis* infection result during visit 0 (base line visit) was taken as starting point for further analysis; *C. trachomatis* was positively identified in 57 females during visit 0, whilst 157 did not have *C. trachomatis* during the same visit.

Sixty-eight of the 219 females came from the Bogota population and 151 belonged to the category "other city"; 40.32% ( $n = 25$ ) of the females infected by *C. trachomatis* at the start of the cohort lived in Bogotá and the remaining 59.68% ( $n = 37$ ) in a population classed as "different to Bogota". [Table 2](#) describes the sociodemographic and clinical variables and risk factors from the information supplied at the start of follow-up, according to *C. trachomatis* infection state. The highest detection rate was obtained during follow-up visit 2 where the highest level of *C. trachomatis* infection was observed ([Fig 1](#)).

The percentage of females infected by multiple types of HPV (77.42%) proving positive for *C. trachomatis* infection was much greater than that for those having just a single HPV infection (22.58%). Regarding *C. trachomatis* coinfection and different types of HR-HPV, it was found that type 16 was present in most females in the cohort (51.59%) compared to the total population, followed by types 18 (40.63%), 45 (36.98%), 58 (27.85%), 31 (23.28%) and 33 (5.47%). The greatest *C. trachomatis* frequency rate for multiple HPV infection was found in HR-HPV type 16 (19.18%) followed by type 58 (17.81%). A statistically significant difference was observed between populations infected by *C. trachomatis* and those without infection regarding females infected by HR-HPV 58 and 45.

For females who began the study without infection, the total time contributed towards the study cohort was 2,281 months; 74.52% ( $n = 117$ ) of them became infected, whilst 25.47% ( $n = 40$ ) remained *C. trachomatis* -free during total follow-up time (around 2 years). Infection rate was 5.1 per 100 people during one month (4.27–6.14 95%CI). [Fig 2](#) shows the probability of the risk of acquiring the infection throughout the follow-up period for the group of women in the cohort who began without having *C. trachomatis* infection. [Fig 3](#) shows the probability of clearing *C. trachomatis* infection in time.

Females who began with infection contributed 587 months at risk to the study cohort, infection rate being 9.7 per 100 people in a month (7.48–12.58 95%CI); 8.06% ( $n = 5$ ) of the females who started infected did not clear the infection during the time the study lasted.

Base line *C. trachomatis* infection frequency was 28% ( $n = 61$ ). There was 85% ( $n = 52$ ) *C. trachomatis* identification by the KL1/KL2 and 29% ( $n = 18$ ) with KL5/KL6 set of primers in

Table 2. The distribution of socio-demographic characteristics and risk factors.

	Chlamydia identification				P
	Negative		Positive		
	n	%	n	%	
<b>Smoker</b>					
No	117	84.78	50	89.29	0.42
Yes	21	15.22	6	10.71	
<b>City</b>					
Bogota	43	27.39	25	40.32	0.062
Other	114	72.61	37	59.68	
<b>Ethnicity</b>					
White	3	1.91	0	0	0.606
Indigenous	1	0.64	1	1.61	
Mestizo	151	96.18	61	98.39	
Black	2	1.27	0	0	
<b>Age on first sexual relationship</b>					
< = 18	112	71.34	38	61.29	0.149
>18	45	28.66	24	38.71	
<b>No of sexual partners</b>					
1	77	49.04	25	40.98	0.057
2–3	56	35.67	18	29.51	
>3	24	15.29	18	29.51	
<b>Family planning method</b>					
No method	69	43.95	28	45.90	0.957
Hormonal	17	10.83	6	9.84	
Other	71	45.22	27	44.26	
<b>STI</b>					
HIV	1	0.65	0	0	0.789
None	124	80.52	48	78.69	
Other	29	18.83	13	21.31	
<b>No of children</b>					
0	8	5.10	3	4.92	0.495
1–2	82	52.23	36	59.02	
3–4	59	37.58	17	27.87	
>4	8	5.10	5	8.20	
<b>No of abortions</b>					
No	93	62	38	64.41	0.746
Yes	57	38	21	35.29	
<b>Multiple HPV infections</b>					
No	38	24.20	14	22.58	0.799
Yes	119	75.80	48	77.42	
<b>HPV16</b>					
No	44	28.03	20	32.26	0.535
Yes	113	71.97	42	67.74	
<b>HPV18</b>					
No	68	43.31	35	56.45	0.079
Yes	89	56.69	27	43.55	
<b>HPV31</b>					

(Continued)

Table 2. (Continued)

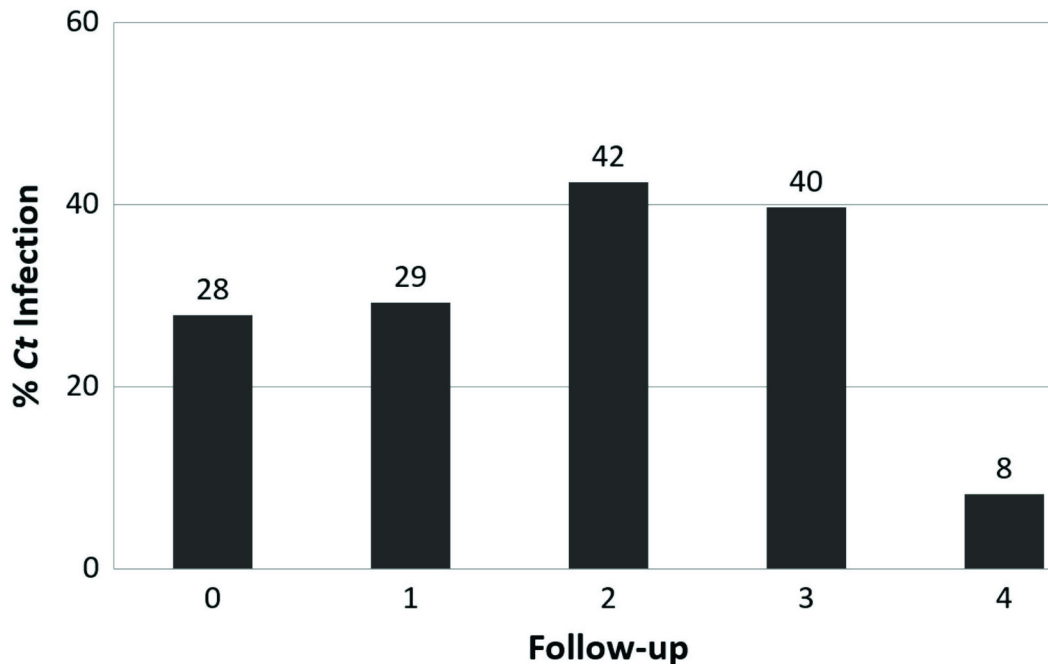
	Chlamydia identification				P
	Negative		Positive		
	n	%	n	%	
<b>No</b>	103	66.88	32	51.61	0.079
<b>Yes</b>	51	33.12	30	48.39	
<b>HPV45</b>					
<b>No</b>	76	48.41	48	77.42	0.000
<b>Yes</b>	81	51.59	14	22.58	
<b>HPV33</b>					
<b>No</b>	145	92.36	60	96.77	0.359
<b>Yes</b>	12	7.64	2	3.23	
<b>HPV58</b>					
<b>No</b>	96	61.15	23	37.10	0.001
<b>Yes</b>	61	38.85	39	62.90	
<b>Number of HPV infecting types</b>					
<b>1</b>	38	24.20	14	22.58	0.119
<b>2</b>	34	21.66	22	35.48	
<b>3</b>	54	34.39	12	19.35	
<b>4</b>	18	11.46	11	17.74	
<b>5</b>	9	5.73	2	3.23	
<b>6</b>	4	2.55	1	1.61	
<b>Cytology<sup>a</sup></b>					
<b>Negative</b>	551	94.67	287	93.79	0.76
<b>ASC-H</b>	1	0.17	0	0.00	
<b>ASC-US</b>	14	2.41	6	1.96	
<b>L-SIL</b>	15	2.58	12	3.92	
<b>H-SIL</b>	1	0.17	1	0.33	
<b>Colposcopy</b>					
<b>Negative</b>	477	89.66	259	90.88	0.296
<b>L-SIL</b>	53	9.96	23	8.07	
<b>H-SIL</b>	1	0.19	2	0.70	
<b>Suggestive</b>	0	0.00	1	0.35	
<b>Unsatisfactory</b>	1	0.19	0	0.00	

<sup>a</sup> ASC-H: atypical squamous cells not exclude H-SIL; ASC-US: atypical squamous cells of undetermined significance; L-SIL: low squamous intraepithelial lesion; H-SIL: high squamous intraepithelial lesion.

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base line data. The detection limit for each set of primers was calculated, the results showing that KL1/KL2 detected lower amounts of *C. trachomatis* DNA (48.2 µg/µL) compared to the KL5/KL6 set of primers (57.6 µg/µL).

Different variables associated with the risk of *C. trachomatis* infection were analysed in this study, such as race, smoking, age on first sexual relationship, the family planning method used, the number of sexual partners, age, the number of children, the relationship of *C. trachomatis* coinfection with multiple HPV infection and the presence or absence of other STI. Amongst these variables, it was observed that females having had the most sexual partners (HR = 6.44: 1.59–26.05 95%CI) or infection with multiple types of HPV (HR = 2.85: 1.22–6.63 95%CI) had the greatest risk of developing *C. trachomatis* infection (Table 3). Moreover, females using a



**Fig 1. Percentage of females infected by *C. trachomatis* per visit.**

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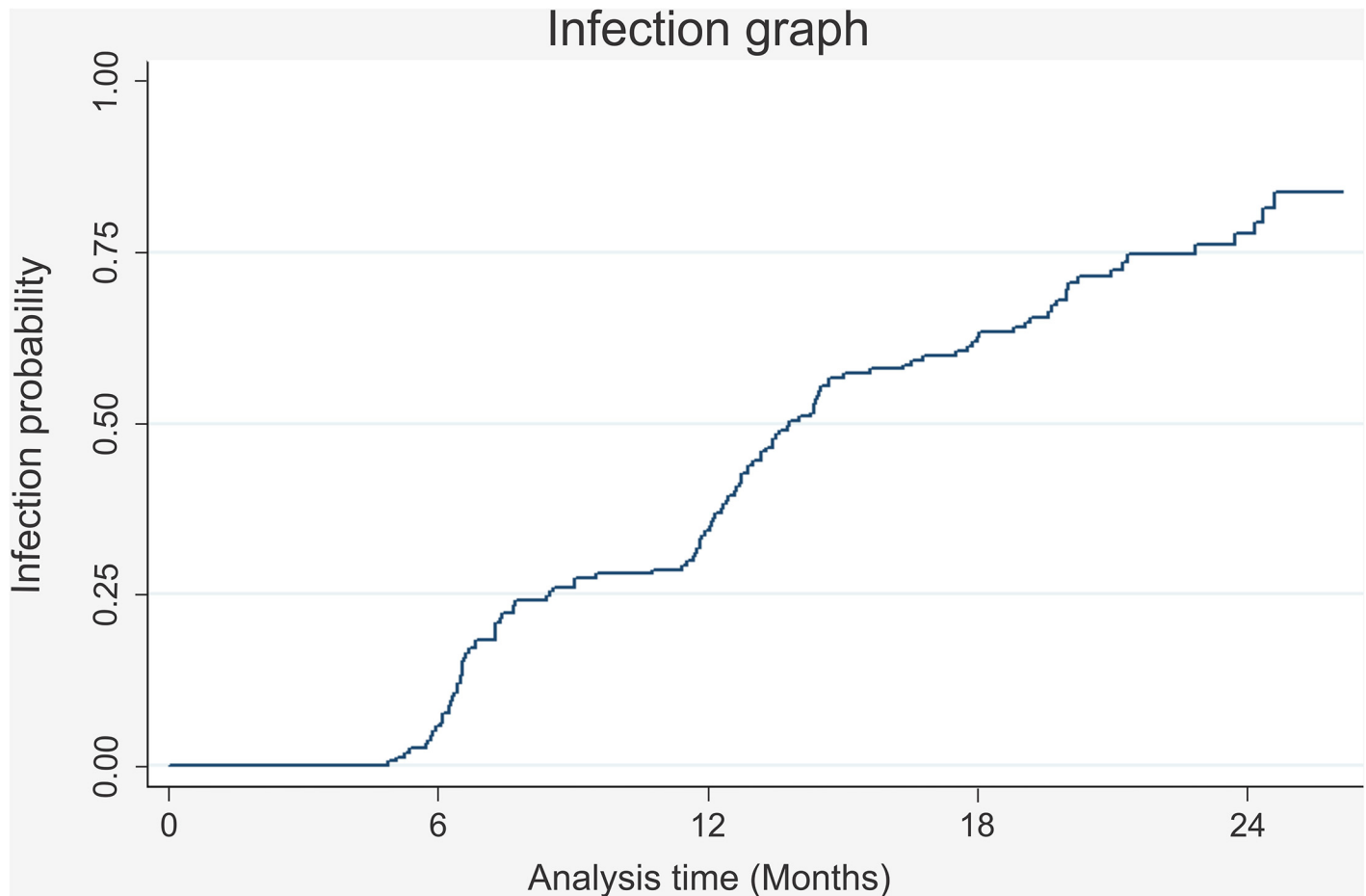
hormonal planning method (HR = 0.33: 0.13–0.79 95%CI) had a reduced risk of developing *C. trachomatis* infection compared to those who did not use any contraceptive method (Table 3).

## Discussion

This study involved a group of 219 females whose ages ranged from 17–71 years old. The estimated base line *C. trachomatis* frequency rate was 28% in a cohort of HPV-infected females from different populations in Colombia, such percentage being higher than that reported in studies in countries like Italy (17%) [24] and Argentina (16.3%) [2] where the samples examined were coinfecting with HPV. The aforementioned 28% was also higher than that reported to date for Colombia (5.0%) [13]; however, it should be stressed that the *C. trachomatis* infection rates in the only Colombian study to date were estimated in a general population of women.

Identifying greater prevalence than that reported in other studies relied on two significant factors. This cohort represented a population at risk since all the females were infected by HPV when the cohort started, meaning that this population was probably engaging in behaviour leading to the risk of acquiring an STI [25]. The second factor was the high sensitivity of the detection technique used here (PCR) [26], as well as two different primer sets being used for *C. trachomatis* identification, thereby increasing the chance of finding positive samples. Previous studies have reported the individual use of the primer sets tested here in samples from HPV-infected women, showing 13% frequency for KL5/KL6 and 34.9% for KL1/KL2 [2, 27].

Regarding the risk factors associated with acquiring *C. trachomatis* infection, an increased number of sexual partners was found, thus agreeing with previous reports describing a similar association not only with *C. trachomatis*, but with almost all STI [28–30]. The fact of having had many sexual partners also implies being involved in risky sexual behaviour thereby facilitating contagion by more than one type of microorganism and hampering clearance of infection [3, 10, 31–33].



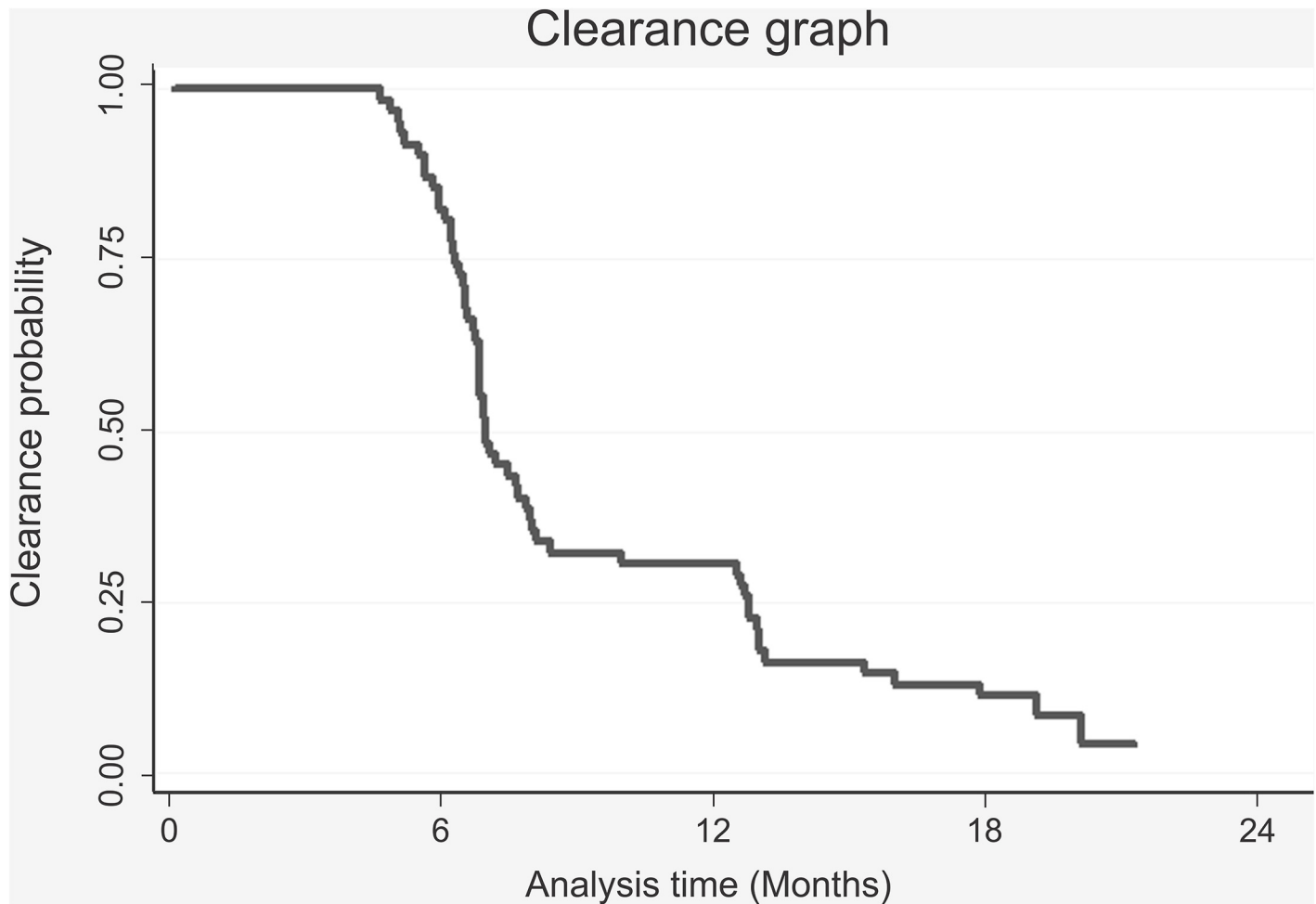
**Fig 2.** The probability of the risk of the women in this cohort acquiring *C. trachomatis* infection as time elapsed.

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Another relevant factor found was infection by multiple HR-HPV types, similar to what has been described in other Latin American countries [10, 27, 34]. The increased risk has been biologically explained by studies showing that *C. trachomatis* infection favours the entry and persistence of multiple HR-HPV types which leads to viral integration, apoptosis inhibition, overexpression of the E6 / E7 oncogenes and cellular transformation [35]. Bearing in mind *C. trachomatis* pathogeny and the tissue damage produced in chronic infections, this epithelium could become affected by various types of HPV virus, leading to multiple infections, as observed in this study. This has been reported in a previous study involving multiple detection of HPV-16 and -18 genotypes [32].

Regarding *C. trachomatis* coinfection with different types of HR-HPV, it was found that types like HPV-16 and -18 occurred most frequently, similar to that reported in a population of female aborigines in the north of Argentina [12]; however, it has been found that HPV-18 and -52 occurs most frequently in the Egyptian population [32]. Such data could suggest that genetic factors might modulate this association or depend on the specific type of HPV distribution in a particular region being analysed; however, further studies are needed regarding the HPV-infected population to elucidate such differences.

The most common *C. trachomatis* coinfection with HPV-HR in the cohort being studied involved type 16. It has been reported in the literature that *C. trachomatis* infection improves



**Fig 3.** The time taken to clear *Chlamydia trachomatis* infection in the cohort of females initially infected by HPV and *C. trachomatis*.

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Ki67 protein expression, this being a marker for cervical epithelium cell proliferation; the same is true for HPV infection, especially that including HPV-16 [12]. It has been found that *C. trachomatis* infection increased HPV-16 expression in CIN I, suggesting that it could modify the activity of this type of virus; it has also been confirmed that *C. trachomatis* infection has increased EGFR and TGF- $\alpha$  expression and this could explain distinct variants of the cervical carcinogenesis mechanism [36].

It is worth mentioning that HR-HPV 58-infected women had the second highest *C. trachomatis* infection frequency in our study which might have been related to a pattern of multiple HPV infection distribution in our population, since a previous report has shown that HPV-58 and -45 are usually present in multiple infections [14].

It could be seen that around a quarter of the HPV-infected females in this cohort had become infected by *C. trachomatis* six months after the study began and that half the population had become infected after one year's follow-up. On the other hand, females who were infected at the start of the cohort required about six months to a year for clearing the infection. This result agreed with a previous study carried out in Bogotá [22] (one of the cities analysed here) which found that 54% of the *C. trachomatis* infected women who did not receive therapy

**Table 3. Cox model.**

Infection risk factors for		
<i>Chlamydia trachomatis</i>	HR (95%CI) <sup>a</sup>	P
<b>Race</b>		
Mestizo	Reference	0.343
Other	1.71 (0.56–5.22)	
<b>Smoker</b>		
No	Reference	0.110
Yes	0.26 (0.04–1.35)	
<b>Age on first sexual relationship</b>		
< = 18	Reference	0.345
>18	1.26 (0.77–2.06)	
<b>Family planning method</b>		
No method	Reference	
Hormonal	<b>0.33 (0.13–0.79)</b>	0.013
Other	0.85 (0.52–1.38)	0.523
<b>No of sexual partners</b>		
1	Reference	
2–3	2.11 (0.63–7.00)	0.221
>3	<b>6.44 (1.59–26.05)</b>	0.009
<b>Age</b>		
< = 35	Reference	
36–45	0.92 (0.50–1.68)	0.804
>45	0.52 (0.26–1.04)	0.067
<b>Children</b>		
0	Reference	
1–2	1.29 (0.43–3.85)	0.643
>3	1.54 (0.49–4.79)	0.455
<b>HR-HPV type coinfection</b>		
= 1	Reference	0.738
2–4	0.90 (0.51–1.60)	
>4	<b>2.85 (1.22–6.63)</b>	0.015
<b>STI</b>		
1	Reference	0.168
2	0.63 (0.33–1.20)	

Values in bold = p < 0.05.

<sup>a</sup> Hazard ratio (HR), adjusted for smoking, city, ethnicity, age on first sexual relationship, number of sexual partners, family planning method, presence of sexually-transmitted infection (STI), the number of children, abortions, confection with HR-HPV, HPV16, HPV18, HPV31, HPV45, HPV33, HPV58, number of HPV infecting types.

doi:10.1371/journal.pone.0147504.t003

resolved the infection in 1 year. No particular variation in infection clearance time was found here when comparing the three geographical populations studied.

Data for asymptomatic *C. trachomatis* infected women in Amsterdam had a person/year spontaneous clearance rate of 44.7%. However, the pertinent study stated that genital *C. trachomatis* infection duration in the absence of treatment had not been completely elucidated, but available long-term studies suggest that a typical untreated *C. trachomatis* infection can last a year or longer before clearance [21, 37].

The present study found that females in the cohort using a hormone-based family planning method had less risk of developing *C. trachomatis* infection; this has been supported by studies in animal models concluding that estradiol reduces the susceptibility to intrauterine *C. trachomatis* infection in rats [38]. Other authors have found that oral contraceptive use has been associated with a faster *C. trachomatis* infection clearance rate [22].

A weakness of this study of a cohort of HPV-infected females was that it lacked parallel screening involving *C. trachomatis* in a population of females who were not infected by this virus for statistically correlating frequency in both groups, thereby contributing towards assessing *C. trachomatis* infection rates in the Colombian population. Determining and analysing the types of *C. trachomatis* in patients having cytological findings would establish whether the relationship of both agents came from mutual potentiation rather than the fact that they share a common transmission route.

## Conclusions

The study provides data regarding the epidemiology of *C. trachomatis*/HPV coinfection in different population groups of Colombian females. Having had more sexual partners and infection by multiple types of HPV is associated with a greater risk of becoming infected by *C. trachomatis*. Using hormonal contraceptives was a factor for the cohort being studied, as significant importance was found regarding an association with lower risk of becoming infected by *C. trachomatis*. This paper contributes towards understanding the natural history of *C. trachomatis* infection.

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## Author Contributions

Conceived and designed the experiments: EMQC DIRC. Performed the experiments: EMQC DIRC. Analyzed the data: EMQC DIRC SCSDL MC RS LDRO JDR MAP. Wrote the paper: EMQC DIRC SCSDL MC RS LDRO JDR MEP MAP.

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# Unveiling the Multilocus Sequence Typing (MLST) Schemes and Core Genome Phylogenies for Genotyping *Chlamydia trachomatis*

Luz H. Patiño<sup>1,2†</sup>, Milena Camargo<sup>2,3†</sup>, Marina Muñoz<sup>1†</sup>, Dora I. Ríos-Chaparro<sup>1</sup>, Manuel A. Patarroyo<sup>3,4</sup> and Juan D. Ramírez<sup>1\*</sup>

<sup>1</sup> Grupo de Investigaciones Microbiológicas-UR (GIMUR), Programa de Biología, Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, Bogotá, Colombia, <sup>2</sup> Ph.D. Programme in Biomedical and Biological Sciences, Universidad del Rosario, Bogotá, Colombia, <sup>3</sup> Molecular Biology and Immunology Department, Fundación Instituto de Immunología de Colombia (FIDIC), Bogotá, Colombia, <sup>4</sup> School of Medicine and Health Sciences, Universidad del Rosario, Bogotá, Colombia

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### \*Correspondence:

Juan D. Ramírez  
juand.ramirez@urosario.edu.co

† These authors have contributed  
equally to this work and are first  
authors

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Multilocus sequence typing (MLST) has become a useful tool for studying the genetic diversity of important public health pathogens, such as *Chlamydia trachomatis* (*Ct*). Four MLST schemes have been proposed for *Ct* (data available from Chlamydiales MLST databases). However, the lack of a sole standardized scheme represents the greatest limitation regarding typing this species. This study was thus aimed at evaluating the usefulness of the four MLST schemes available for *Ct*, describing each molecular marker's pattern and its contribution toward a description of intra-specific genetic diversity and population structure. The markers for each scheme, showed a variable power of discrimination, exhibiting in some cases over estimation in the determination of Sequence Types (STs). However, individual analysis of each locus's typing efficiency and discrimination power led to identifying 8 markers as having a suitable pattern for intra-specific typing. Analyzing the 8 candidate markers gave a combination of 3 of these loci as an optimal scheme for identifying a large amount of STs, maximizing discrimination power whilst maintaining suitable typing efficiency. One scheme was compared against core genome phylogenies, finding a higher typing resolution through the last approach. These results confirm once again that although complete genome data, in particular from core genome MLST (cgMLST) allow a high resolution clustering for *Ct* isolates. There are combinations of molecular markers that could generate equivalent results, with the advantage of representing an easy implementation strategy and lower costs leading to contribute to the monitoring and molecular epidemiology of *Ct*.

**Keywords:** MLST, MLST-genotyping, sequence type (ST), schemes, *chlamydia*

## INTRODUCTION

*Chlamydia trachomatis* (*Ct*) has been the species having the greatest clinical and epidemiological importance; it infects the human genital-urinary tract, being the most common bacterial sexually-transmitted infection (STI) worldwide (WHO, 2011; O'Connell and Ferone, 2016). Alternatively, *Ct* can infect the ocular mucosa, being responsible for the development of trachoma, the main cause

of infectious blindness around the world (Dean et al., 2013; Shao et al., 2013; Anaene et al., 2016; Lallemand et al., 2016). In 2012, the WHO reported around 131 million new *Chlamydia* infections worldwide, the 60 percent of the cases were presented in developed countries, however most of them go unnoticed without diagnosis or adequate treatment (Newman et al., 2015; WHO, 2016). Until now *Ct* constitutes the species with most interest due to the impact on human health (mainly on sexual and reproductive health) (Bom et al., 2011; Christerson and Herrmann, 2012).

*Ct* has a single circular chromosome having more than 1 million base pairs (bp) as well as a 7.5 kb highly conserved plasmid having multiple copies within a cell (Tam et al., 1992; Seth-Smith et al., 2013; Nunes and Gomes, 2014; de Vries et al., 2015; Pawlikowska-Warych et al., 2015; Anaene et al., 2016; Jelocnik et al., 2016). This species is characterized by conserved genomes and by the low level of genetic diversity among variants (<2% of the genome). However, it presents some regions with high events of recombination and nucleotide diversity (Joseph and Read, 2012). Molecular differences between strains have been seen to be associated with its tropism and geographical distribution; genetically different strains have been identified as infecting various populations [men having sex with men (MSM), heterosexuals and bisexuals] (Gravningen et al., 2012). Such characteristics have led to the use of different typing techniques enabling a strains' tissue tropism to be determined, identifying and differentiating new or persistent infections, understanding transmission dynamics, and monitoring how specific clones evolve (Rawre et al., 2017).

Serotyping has traditionally been used for typing *Ct*; it uses specific antibodies directed against the outer membrane protein (MOMP). However, this technique is considered laborious, takes too long and has low sensitivity (Nunes and Gomes, 2014). Some molecular techniques used for typing *Ct* have been restriction fragment length polymorphism (RFLP), DNA hybridisation-based techniques, polymerase chain reaction (PCR) and DNA microarrays based on analysis of the *ompA* gene (encoding MOMP) (Stothard, 2001; Quint et al., 2007; Pannekoek et al., 2008; Pedersen et al., 2009; Ruettinger et al., 2011; Xia and Xiong, 2014; Gallo Vaulet et al., 2016). These have led to 19 variants being identified (Pannekoek et al., 2008), grouped into 3 clusters; one includes variants L1-L3 and L2a, associated with Lymphogranuloma venereum (LGV), another covers variants A, B, Ba, and C, associated with trachoma and another covers variants D-K, Da, Ga, Ia, and Ja, associated with genital-urinary infections (Pedersen et al., 2009; Herrmann et al., 2015; Sherchand et al., 2016; Petrovay et al., 2017).

The low discrimination power of some of the techniques mentioned above and their multiple disadvantages have led these techniques to be replaced by other typing methods especially those based on sequencing, which are much more specific and enable *Ct* intra-specific typing (de Vries et al., 2015). Among these techniques emerges the Multilocus Sequence Typing (MLST) (Klint et al., 2007; Pedersen et al., 2008; Bom et al., 2011; Xia and Xiong, 2014; de Vries et al., 2015) that has provided a portable, reproducible and scalable typing system and is performed easily by different laboratories (Urwin and

Maiden, 2003). Additionally, recent studies using whole-genome sequencing (WGS), have allowed expanding the knowledge about the epidemiology, evolutionary history and diversity of members of *Ct* based on recent approaches defined as core genome MLST (cgMLST) (Harris et al., 2012; Rawre et al., 2017). Despite the WGS (Whole genome MLST and cgMLST) has demonstrated to be a tool with a high discriminatory power. This technique presents some disadvantages due to its higher costs and requirement of big computational capacity (Tsang et al., 2017; Versteeg et al., 2018).

Several MLST schemes have been described to date for genotyping *Ct* and have been designed with different purposes (**Supplementary Table S1**); one has been designed to analyse evolutionary changes over time and its usefulness for comparison of strains from different species (Pannekoek et al., 2008) and others for describing *Ct* intra-taxa variability (Dean et al., 2009), one such based on seven housekeeping genes (*C. trachomatis* MLST scheme) and another on five highly variable regions (*C. trachomatis* - Uppsala MLST scheme) (Grieshaber et al., 2006; Klint et al., 2007) has been designed to discriminate only *C. trachomatis* strains for epidemiological purposes. Finally, there is also the plasmid loci MLST scheme (<https://pubmlst.org/chlamydiales/>), which makes use of regions in the 8 putative open reading frames encoded by a 7.5 Kbp plasmid in most *Ct* isolates (Rockey, 2011), however there is not enough information associated with its use.

In spite of MLST schemes' many advantages and clinical applications, there is currently no single standardized scheme for typing *Ct*. This study was thus aimed at analyzing all the MLST schemes available for *Ct* (Chlamydiales, *C. trachomatis*, *C. trachomatis*—Uppsala and plasmid loci) to determine the schemes' robustness (resulting from combining multiple loci), as well as the molecular markers independently, and evaluate their usefulness for describing intra-specific genetic variability. It was also aimed at evaluating how such information can describe *Ct* genetic population structure, representing an indicator of transmission dynamics and signals leading to the variability of this group of organisms. We finally compared the results with phylogenies retrieved from cgMLST (Tsang et al., 2017). The study sought to identify the best combination of molecular markers enabling *Ct* isolate typing, maintaining suitable intra-species discrimination power using a core genome MLST as reference. Finally, it is important to mention that although the data obtained in each scheme come from different studies and were performed with different purposes, they represent to date the dataset currently available worldwide for *Ct*.

## MATERIALS AND METHODS

### Data Retrieval

All data were obtained from public databases for molecular typing and microbial genome diversity (<https://pubmlst.org/>) (Jolley and Maiden, 2010); such databases (curated and public access) included the Chlamydiales MLST website where descriptive data set was accessed (related to geographical origin, characteristics regarding source and traditional classification, etc.), isolates reported to date (<https://pubmlst.org/>).

org/bigsdbs?db=pubmlst\_chlamydiales\_isolates) as well as downloading sequences covering all known diversity for the Chlamydiales species and *Ct* variants via the locus/sequence definitions database ([https://pubmlst.org/bigsdbs?db=pubmlst\\_chlamydiales\\_seqdef](https://pubmlst.org/bigsdbs?db=pubmlst_chlamydiales_seqdef)).

The date of the last update of the database at the moment of conducting the analyzes was 03-15-2017.

Four MLST schemes were found in the Chlamydiales MLST database, the first includes the genes *gatA*, *oppA*, *hflX*, *gidA*, *enoA*, *hemN*, and *fumC* (Pannekoek et al., 2008). This scheme is the most used for isolates of the order Chlamydiales, because it allows discrimination at the species level. However, when considering the objective of the present work, exclusively the sequences of *Ct* were selected and used for the analysis conducted for the group that is referred to as Scheme A. The three remaining schemes focus exclusively on the typing of *Ct* isolates: Scheme B *C. trachomatis* MLST scheme (*glyA*, *mdhC*, *pdhA*, *yhbG*, *pykE*, *lysS*, and *leuS*) (Dean et al., 2009), Scheme C *C. trachomatis* - Uppsala MLST scheme (*CT058*, *CT144*, *CT172*, *hctB*, and *pbpB*) (Klint et al., 2007) and Scheme D the plasmid loci MLST scheme (CHLAM0895, CHLAM0896, CHLAM0897, CHLAM0898, CHLAM0899, CHLAM0900, CHLAM0901, and CHLAM0902). The seven genes used in Schemes A,B were housekeeping genes, whilst Scheme C's five genes were considered hypervariable. The four MLST schemes made use of 27 molecular markers constituting the dataset for subsequent analysis. **Figure 1** and **Supplementary Table S1** give information regarding the genes included in the MLST schemes.

## Descriptive Analysis

Chlamydial species' descriptive analyses were conducted from the breakdown section's exportable dataset ([https://pubmlst.org/bigsdbs?db=pubmlst\\_chlamydiales\\_isolates&page=job&id=BIGSdb\\_057458\\_1492173438\\_56586](https://pubmlst.org/bigsdbs?db=pubmlst_chlamydiales_isolates&page=job&id=BIGSdb_057458_1492173438_56586)). This dataset gave information/variables regarding the hosts from which each isolate was obtained concerning age, country, region, sample source, gender, host and *Ct* variant isolated. Variables were treated as categorical and described in terms of frequency and percentage; 95% confidence intervals were used when events of interest were calculated (bootstrap). Chi<sup>2</sup> or Fischer's exact tests were used for estimating differences regarding distribution, depending on the nature of the data. STATA12<sup>®</sup> software was used for all statistical analysis (0.05 significance for all hypothesis testing).

## Molecular Markers Characteristics

The allele sequences reported for each molecular marker were downloaded in FASTA format via the option, 'Download allele sequences' ([https://pubmlst.org/bigsdbs?db=pubmlst\\_chlamydiales\\_seqdef&page=downloadAlleles&tree=1](https://pubmlst.org/bigsdbs?db=pubmlst_chlamydiales_seqdef&page=downloadAlleles&tree=1)), providing alignments for the different schemes available. The sequences for all allele profiles reported for each MLST scheme were downloaded via the option, "Export allele sequences in XMFA/concatenated FASTA formats—Chlamydiales locus/sequence definitions" ([https://pubmlst.org/bigsdbs?page=plugin&name=SequenceExport&db=pubmlst\\_chlamydiales\\_seqdef](https://pubmlst.org/bigsdbs?page=plugin&name=SequenceExport&db=pubmlst_chlamydiales_seqdef)), selecting all loci included in the MLST

scheme. The ClustalW algorithm was used for initially comparing all sequences via multiple alignment (Thompson et al., 2002) to identify the percentage of identity regarding alleles' total length.

Each marker's nucleotide composition was then analyzed for identifying the amount of variable sites between the reported alleles and compared with those sites considered as informative according with parsimony principle (positions containing at least two types of nucleotides having a minimum frequency of two). This rate, named here "informative indices" was calculated to provide comparable data regarding the molecular markers.

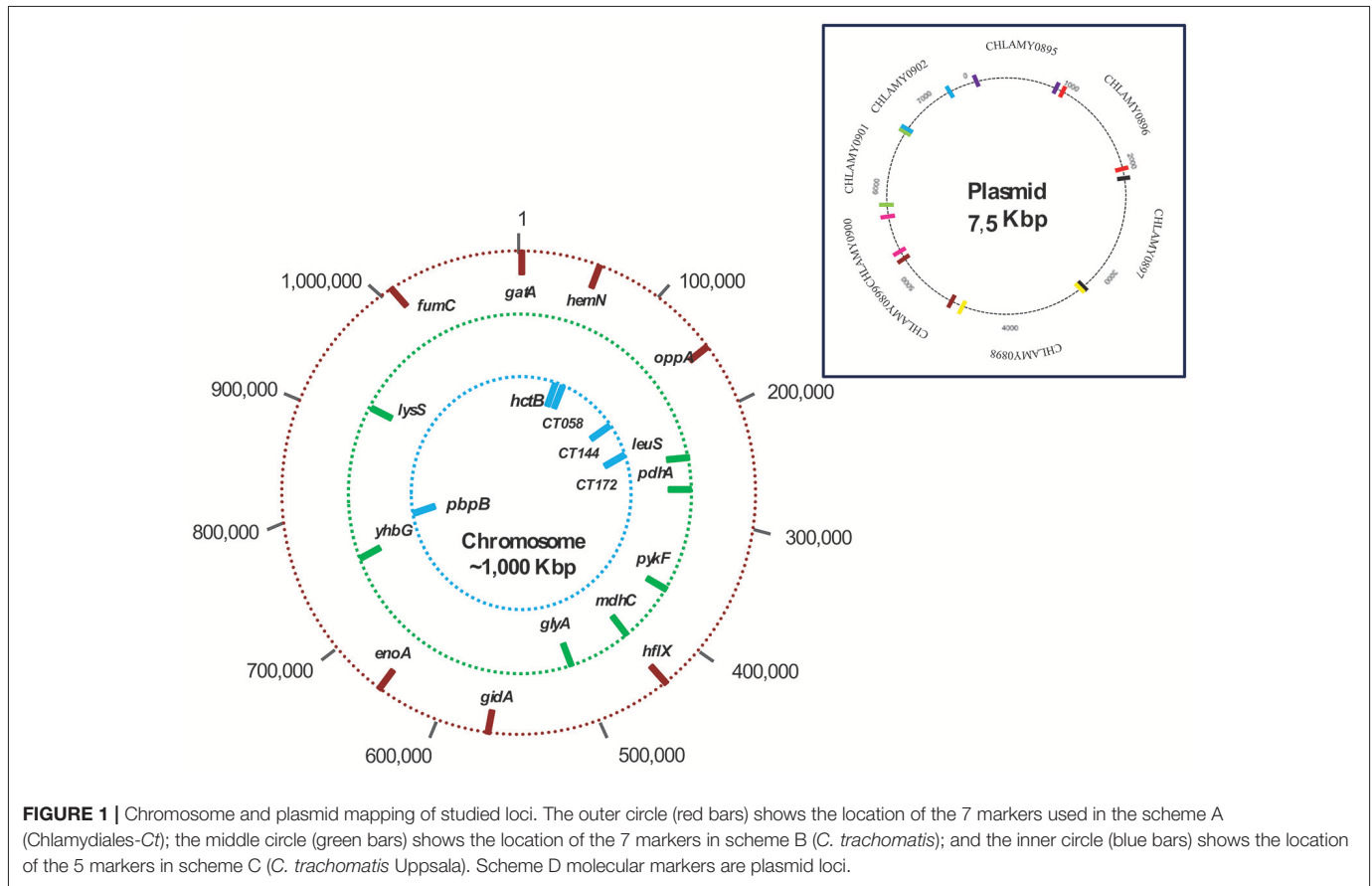
## Genetic Diversity Indices

The sequences were aligned using MUSCLE (alleles reported for each marker and total STs concatenated sequences), later were evaluated to identify insertion and deletion (indels) events, which might have altered the length of the sequences to be analyzed. Once the indels were identified, those were edited to eliminate the gaps. DnaSP software (v5) was then used for analyzing verified alignments' genetic diversity: total amount of mutations (Eta), amount of haplotypes (h), haplotype diversity (Hd), defined as the probability that two randomly chosen haplotypes would be different, nucleotide diversity ( $\pi$ ), representing the average number of nucleotide differences per site between two randomly chosen DNA sequences, Theta (per site) from Eta, Theta (per site) from S (ThetaW), where Eta (h) represented the total amount of mutations and S was the amount of segregating (polymorphic) sites and average number of nucleotide differences (k). Some calculated indices [Haplotype diversity and Theta (per site) from S (ThetaW)] are reported with their respective 95% confidence intervals.

## Phylogenetic Analyses

Phylogenetic reconstructions were made from the alignments regarding the total length of molecular markers included in each MLST scheme (for each gene and concatenated sequence for each scheme). The jModelTest v.2.1.10 (Lemey, 2009; Darriba et al., 2012) was applied on all the alignments, considering the Akaike Information Criterion "AIC" (Alfaro and Huelsenbeck, 2006), in order to identify the best substitution model for phylogenetic reconstructions. Considering that Tamura-nei (TrN) (Tamura and Nei, 1993) was the model that consistently presented low AIC results, this was applied for all subsequent analyses.

Bootstrap method (BT; with 1,000 replicates) was used for evaluating the nodes' robustness, considered a well-known statistical tool for approximates of the variance of the data under the real model of sequence evolution (Wróbel, 2008). Each phylogenetic reconstruction was analyzed for identifying the number of clusters produced, defined as nodes having  $\geq 80.0\%$  BT values. A second screening was necessary for molecular markers where no clusters exceeding this cut-off point were identified, reducing  $\geq 60.0\%$  bootstrap cut-off values. BT replicates were increased to 10,000 when  $\leq 1$  cluster was identified, during the preliminary phylogenetic analysis (i.e., Scheme B). It has classically been reported that more than 1,000 replicates are needed to attain  $\pm 1\%$  accuracy for bootstrap proportions of 95% or higher (Hedges, 1992). Increasing the number of BT replicates would produce greater resolution power, contributing



in the evaluation of statistical significance of the relative validity of phylogenetic reconstructions (Müller, 2005; Deng et al., 2013). Homologous genes in closely related species were identified for each marker as outgroup for the phylogenetic reconstructions. Homologous genes in *Parachlamydiaceae acanthamoebae* were used in Schemes A,B and homologous regions in *C. muridarum* were included in Schemes C,D. FastTree version 2.1.9 Double precision (Price et al., 2010), was used to conduct phylogenetic trees based on molecular markers.

ST classification for each marker/scheme was graphically represented via allele plot, assigning a color to each well-supported cluster in each phylogenetic reconstruction. It was then determined to which each ST belonged (assigning a corresponding color). The number of colors in allele plots thus represented the amount of clusters discriminated by each molecular marker as reported elsewhere (Muñoz et al., 2017). Molecular Evolutionary Genetics Analysis software (MEGA7 version 7) was used for all alignments and phylogenetic reconstructions (Kumar et al., 2016).

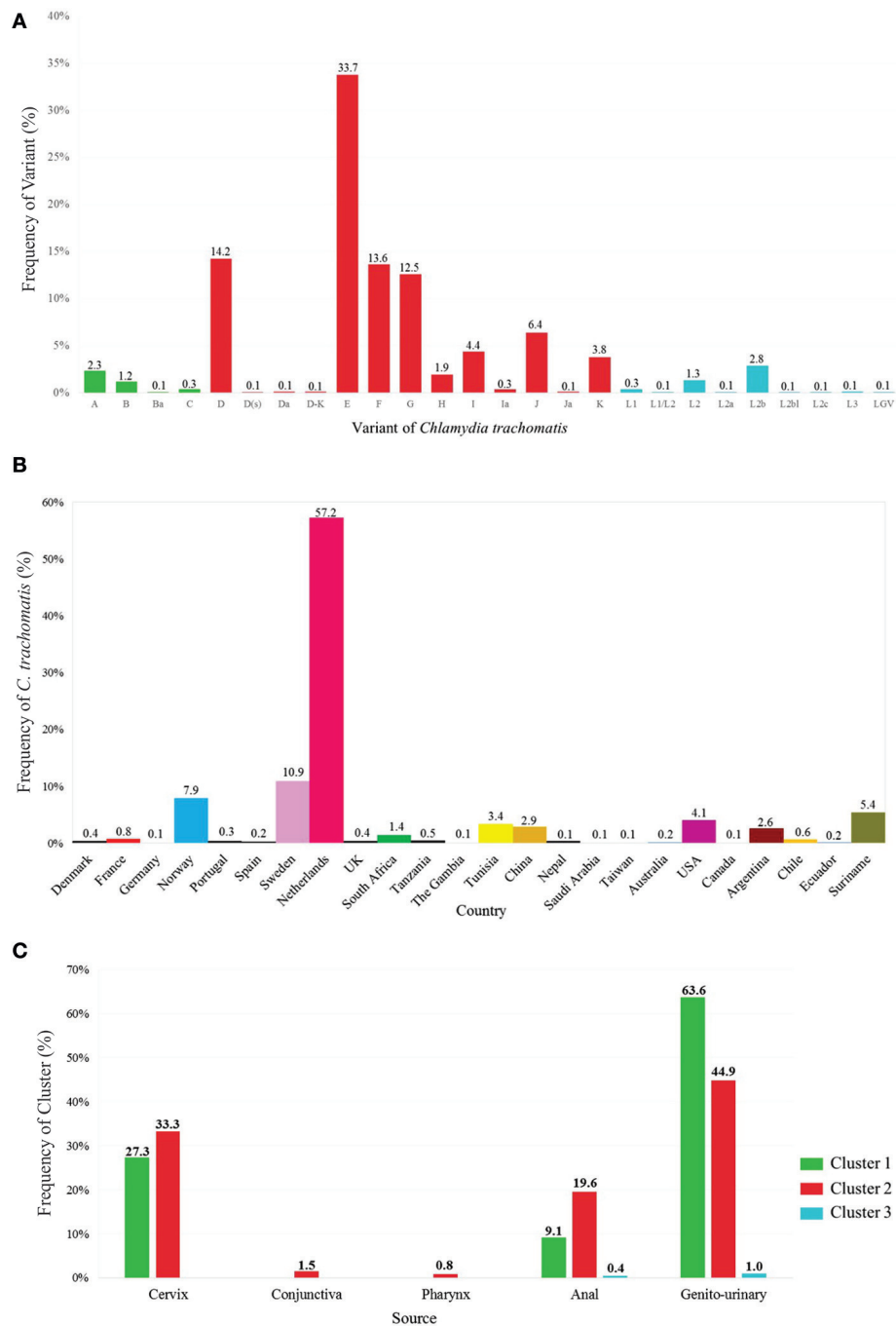
### Multilocus Sequence Analysis (MLSA)

The allele profiles for the STs reported for each MLST scheme were analyzed to identify related ST groups (clone complexes—CC) and make evolutionary inferences by identifying founder genotypes (ST) for each CC identified via each MLST scheme; eBURSTv3 software was used for such analysis (Feil et al., 2004).

Parallel to this, phylogenetic networks were developed using the Neighbor-net method available in the SplitsTree4 package (version 4.14-4) for identifying rearrangements to which the molecular markers included in each MLST scheme (loss and duplication events, hybridisation, horizontal gene transfer or recombination) could undergo (Huson and Bryant, 2006).

### Comparison of MLST Schemes With Whole Genome Sequencing Data

An additional analysis was conducted using WGS data, considering it as the most robust source of data to evaluate the clustering of isolates and to plausibly depict the best-fit *Ct* typing Scheme. A set of public available genomes was downloaded and then used to compare the clustering obtained by the multiple MLST schemes against wgs typing. The data set was obtained from the following databases: PATRIC 3.5.11 ([https://www.patricbrc.org/view/GenomeList/?and\(keyword=chlamydia,keyword\(trachomatis\)\)#view\\_tab=genomes](https://www.patricbrc.org/view/GenomeList/?and(keyword=chlamydia,keyword(trachomatis))#view_tab=genomes)), NCBI Sequence Read Archive—SRA (<https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=announcement>), European Nucleotide Archive—ENA (<https://www.ebi.ac.uk/ena/data/search?query=chlamydia+trachomatis>), National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database (<http://www.ncbi.nlm.nih.gov/refseq/>) and Wellcome Sanger Institute (<http://www.sanger.ac.uk/resources/downloads/bacteria/chlamydia->



**FIGURE 2** | Description of *Ct* variants in isolates reported in Chlamydiales MLST databases. **(A)** *Ct* variant frequency ( $n = 3,242$ ): Green shows cluster 1 variants (associated with ocular infections), red shows cluster 2 variants (associated with urogenital infections) and blue shows cluster 3 variants (associated with *Lymphogranuloma venereum*). **(B)** *Ct* distribution according to country ( $n = 3,242$ ). **(C)** *Ct* cluster distribution according to sample source ( $n = 2,194$ ).

trachomatis.html). All databases consulted are curated and freely accessible.

“*Chlamydia trachomatis*” was used as a search term in the different databases. For each match found, the multi-file assembly was downloaded. For the genomes found in more than one database, only one report was considered. Once the complete

genomes were obtained, the quality control of the raw data was carried out using the GenomeQC Filter (v1-5.pl), which considers the following parameters: (i) a maximum number of 400 contigs allowed, (ii) a maximum genome size of 8 Mb, and (iii) a similarity of at least 95% between 16S ribosomal RNA (16SrRNA) sequences. The genomes with poor quality

were excluded. In parallel, the extracted 16S rRNA sequence was used, both for the verification of taxonomic allocation using the SINA Alignment Service tool, available in SILVA rRNA gene database (Quast et al., 2013), SILVA database, and for the generation of a phylogenetic reconstruction based on 16SrRNA, in order to verify the clustering within the same species.

The genomes that passed the quality tests were used to predict the ST considering the Chlamydiales scheme, using the mlst-2.10 package (Seemann, 2018). This tool was used to predict the allelic profiles of the set of genomes evaluated, both by Scheme A, which is predetermined within the databases included in the mlst-2.10 package, and by Schemes B,C, which were added to the databases, using the information available in Chlamydiales MLST database. The concatenated sequence of the determined ST was used to construct a multiple alignment and to carry out a phylogenetic reconstruction.

In parallel, the set of selected genomes was annotated using Prokka version 1.13 (Seemann, 2014), as a preliminary step for determining the pangenome of the analyzed data set using Roary (by means of a blastp percentage identity of 95% and a core definition of 99%) (Page et al., 2015). A phylogenetic tree based on the core genome of the analyzed data set was inferred, which was considered as a 'reference' of the clustering ('core genes' are shared by more than 95% of the data included in the analysis and represent the most robust data set for the generation of high resolution phylogenies) (Sentausa and Fournier, 2013; Wang et al., 2015). Additionally, the multi FASTA alignment file of core genome was used to identify the Single Nucleotide Polymorphisms, using the SNP-sites program (Page et al., 2016). Phylogenetic reconstructions from core genome SNPs were conducted to compare the clustering of the schemes against the markers herein evaluated. For phylogenetic reconstructions based on core genome and core genome SNPs, the alignments were analyzed using the Randomized Axelerated Maximum Likelihood (RAxML v.8) method. The clustering of the set of isolates was then evaluated through the comparison of the obtained phylogenetic reconstructions.

## Marker Usefulness for Intra- Specific Typing

MLSTest software was used for calculating the number of alleles and polymorphisms, typing efficiency (TE) and discriminatory power (DP), using Simpson's index (and 95% IC) (Tomasini et al., 2013). Each marker's alignment was used as data source, including alleles for all STs reported here. MEGA7 software (Nei and Gojobori's method) was used for calculating the ratio of non-synonymous (dN) to synonymous (dS) substitutions per nucleotide site (dN/dS) for inferring the type of selection to which each molecular marker was exposed. TE and DP were described in terms of means and standard deviations (SD) for the Schemes. Markers having high TE and DP (within the 75 percentile) were then analyzed regarding scheme optimisation for identifying the optimum number of loci required. Such analysis involved the sequences for 179 isolates constituting the only group having information for the 3 MLST schemes

(Schemes A–C), considering that Scheme D is used *in silico*, was excluded from this analysis.

## RESULTS

### Descriptive Analysis

Initial analysis of the Chlamydiales MLST database revealed information available for 4,024 isolates, including those having a typing result by any of the 4 MLST schemes evaluated here. The aforementioned isolates had been recorded from 1957 to 2017 and had been reported in the database up to 2017-03-15 (last update taken for data analysis). Of the total data reported, 3,691 correspond to *Ct*, this data set was used for describing distribution profiles for *Ct* (for clusters, variants or STs), according to age, gender and/or sample source.

Geographical distribution pattern of *Ct* was analyzed regarding a set of 3,133 data set isolates; 78.1% ( $n = 2,448$ ) of the isolates were reported in Europe (Austria, Denmark, France, Germany, Norway, Poland, Portugal, Spain, Sweden, Switzerland, the Netherlands and the United Kingdom), 8.8% ( $n = 277$ ) from South America (Argentina, Chile, Ecuador and Suriname), 5.4% ( $n = 168$ ) from Africa (South Africa, Tanzania, the Gambia and Tunisia), 4.1% ( $n = 129$ ) from Asia (China, Nepal, Russia, Saudi Arabia and Taiwan), 3.4% ( $n = 105$ ) from North America (Canada and the USA), and 0.2% ( $n = 6$ ) from Oceania (Australia). The highest percentage of isolates was reported from the Netherlands (57.2%;  $n = 1,793$ ), followed by Sweden (11%;  $n = 342$ ), and Norway (7.9%;  $n = 249$ ). The remaining countries had <5% isolates (**Supplementary Figure S1**). As most available data concerned *Ct*, some characteristics of interest for this species were described, i.e., gender, sample source, variant, worldwide distribution, sampling site of the clusters analyzed and age (**Supplementary Table S2** and **Supplementary Figure S2**).

### Describing the MLST Schemes

The Chlamydiales MLST Databases contained 16,019 sequences at cut-off date. This included those used for classifying each MLST scheme and led to identifying 75 STs for Scheme A, 44 STs for Scheme B, 520 STs for Scheme C, and 47 STs for Scheme D. Regarding the amount of STs described for each MLST scheme for each *Ct* variant, there were more Cluster 2 (associated with genitourinary infections) variants in all schemes, contrasting with that observed for variants related to LGV (including the least amount of STs) (**Supplementary Table S3** and **Supplementary Figure S3**). **Supplementary Figure S3** describes the amount and frequency of each ST per variant, discriminating classification by each MLST scheme.

Initial descriptive analysis of the sequences reported for each molecular marker led to determining that the number of alleles reported ranged from 7 (for *glyA*, *pdhA*, and *pykF* genes used for Scheme B) to 92 (*hctB* used for Scheme C). Sequence identity analysis of alleles reported for each molecular marker showed that Scheme A shows identity percentages between 39.2 and 99.1% (being *hemN* the one that showed less percentage of identity and *gatA* the one with the highest one). Greater than 95% identity was observed

for all genes in Scheme B whilst markers in Scheme C were highly heterogeneous, ranging from 14.7% (marker CT172) to 88.6% (CT086). Identity values ranging from 83.6% (marker CHLAM0895) to 98.3% (CHLAM0897) were found for Scheme D. Sequence identity percentages can be consulted in **Supplementary Figure S4-A**.

Considering that not all variable sites were informative (according to parsimony principle), the rate between the number of variable sites vs. informative sites was calculated, with the aim of generating a comparative data (named “informative index” here) between molecular markers. The results were inversely proportional to sequence identity patterns (genes having the highest identity percentages displayed the lowest informative indexes). The most interesting informative index pattern was for Scheme D, in which markers CHLAM0895 and CHLAM0898 had the maximum result (1.0000), indicating that all variable sites could be considered as informative, contrary to what happened with marker CHLAM0900, that showed a null result (0.000), provided none of the variable sites were informative. **Supplementary Figure S4-B** describes the number of variable sites compared to informative ones for all molecular markers.

### Analyzing Genetic Diversity

**Supplementary Table S4** reports the intra-species genetic diversity indices calculated for each MLST scheme (molecular markers and concatenated sequences). **Figure 3** gives a graphical representation of nucleotide diversity compared to haplotype diversity indices for each set of data. Nucleotide diversity values were <0.2222 for all schemes concerning independent analysis for each marker and concatenated sequence. However, when comparing MLST schemes, the results showed that the highest nucleotide diversity indices were present in Scheme C, marker CT172 (0.18962) and concatenated sequences (0.22224). The rest of the markers showed nucleotide diversity <0.0568.

The greatest nucleotide diversity for Scheme B was 0.03129 (*hemN*) and 0.00950 (CHLAM0899) for Scheme D. Regarding haplotype diversity, it was found that Schemes A,B,D had values close to 1.000, differently to Schemes A,C where heterogeneous patterns were observed, being lower for Scheme A [0.328 (*hemN*) to 0.771 (*gidA*)] compared to Scheme C [0.801 (CT172)]. **Supplementary Table S4** gives all genetic diversity indices calculated for each MLST scheme.

### Analyzing Clonal Complexes (CC)

The concatenated sequences for each MLST scheme evaluated were used for depicting CC clustering patterns via the eBURST algorithm. The results showed that the STs identified via Scheme A led to 3 CC and 6 singletons being identified; CC1 and CC2 had most STs (36 and 26 STs, respectively), their founder STs (ST13 and ST4, respectively) being associated with urogenital infections. Founder CC3 (including 7 ST) was associated with LGV (ST44).

eBURST analysis of Scheme B, 3 CC and 11 singletons were identified; CC1 had most STs (19 STs), founder ST 19 being associated with urogenital infections, followed by CC2 and CC3 having the same amount of STs (7) and whose founder ST 11 has been associated with urogenital lesions and 34 with

trachoma. Regarding Scheme C was grouped into 15 CC and 55 singletons, having two CC mainly consisting of 241 and 167 STs, respectively. Founder ST for these majority groups were 56 and 106, both being associated with urogenital infections. When evaluating Scheme D, 6 CC and 12 singletons were identified, CC1 having most STs (12 STs) which were associated with urogenital lesions. **Figure 4** describes most of CC organization for each MLST scheme. **Supplementary Table S5** gives complete eBURST analysis results for each MLST scheme.

### Clustering Each MLST Scheme/Molecular Marker

Phylogenetic reconstructions of the sequences for all STs reported for each MLST scheme were made for each molecular marker and concatenated sequence; they were then used as the basis for determining their discrimination power (represented in allele plot, **Figure 5**). Regarding the discriminatory power, the results showed that Scheme A identified 2–4 well-supported clusters, and the marker *hemN* providing most clusters.

Analyzing Scheme B highlighted all markers’ (1–4 cluster) low discrimination power, the *mdhC* gene being the marker having the greatest discrimination power (the only one having 4 clusters). These results led to identifying these markers’ low polymorphism. It was found that Scheme C had high discrimination power; 20 well-supported clusters and 3 outliers were found in phylogenetic reconstruction based on concatenated sequences. This pattern was confirmed by grouping by markers such as CT144 (12 clusters) and *pbpB* (7 clusters). Interestingly, it was found that marker CT172 only produced 1 cluster which included all the STs.

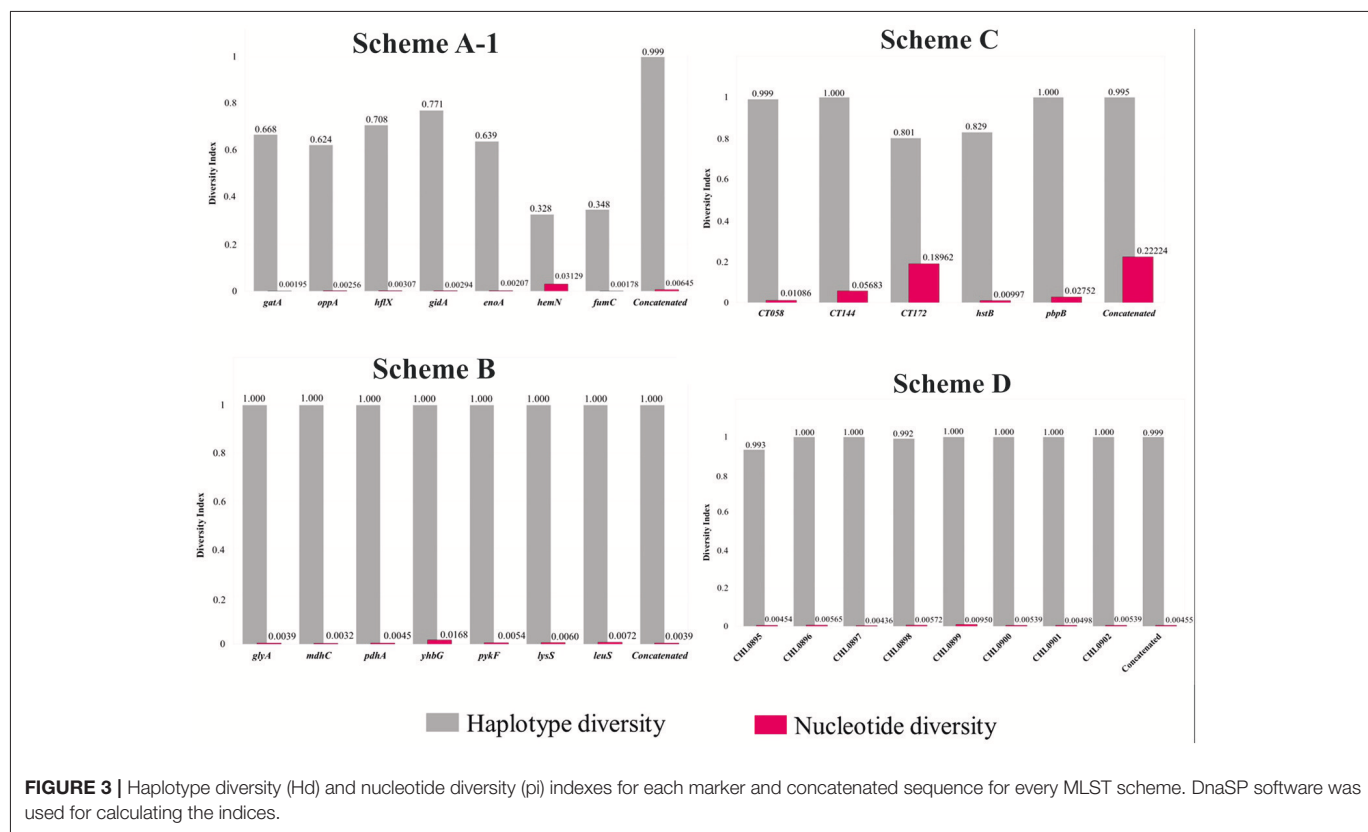
Analyzing Scheme D led to determining the high discrimination power of this scheme’s loci (3–8 clusters), loci CHLAM0895, CHLAM0898, and CHLAM0899 being the markers having the greatest discrimination power.

The allele plot patterns showed less clusters (less diversity of colors). These were observed for schemes directed toward *Ct* typing, especially Scheme B, where a single cluster (in green) predominated, followed by Scheme A, where only some STs belonged to a second cluster with relative frequency (in red). In contrast, the Scheme D was the only MLST scheme having a pattern displaying many clusters (represented by the greatest diversity of colors) (**Figure 5**).

SplitsTree software was used for constructing phylogenetic networks to verify molecular rearrangements regarding the molecular markers used in each scheme (Neighbor-net algorithm). Concatenated sequence analysis for each scheme revealed reticulation events, mainly for Scheme C. In spite of preliminary indications of recombination identified for Scheme D, no reticulation events were found in the allele plot for the phylogenetic network. Finally, no marked reticulation events were observed for Schemes A,B (**Supplementary Figure S5**).

### WGS Phylogenetic Reconstructions’ Comparison

In total, 243 complete genomes were found in the different databases consulted. The quality control analyses led to



the exclusion of 83 genomes, because 13 of them showed a contig count above the established limit (between 419 and 15,664 contigs) and in the remaining 70 genomes, the 16SrRNA sequence was not identified. The additional step for verifying the taxonomic allocation of the set of genomes using the SINA Alignment Service tool, available in the SILVA rRNA gene database (Quast et al., 2013), showed that one of them corresponded to *Mycoplasma* (BioSample Accession: SAMEA1398231). This finding was confirmed in the phylogenetic reconstruction based on 16SrRNA that was carried out in parallel, where the sequence of this genome (813.61), was the only one that clustered outside the *Ct* cluster (**Supplementary Figure S6**).

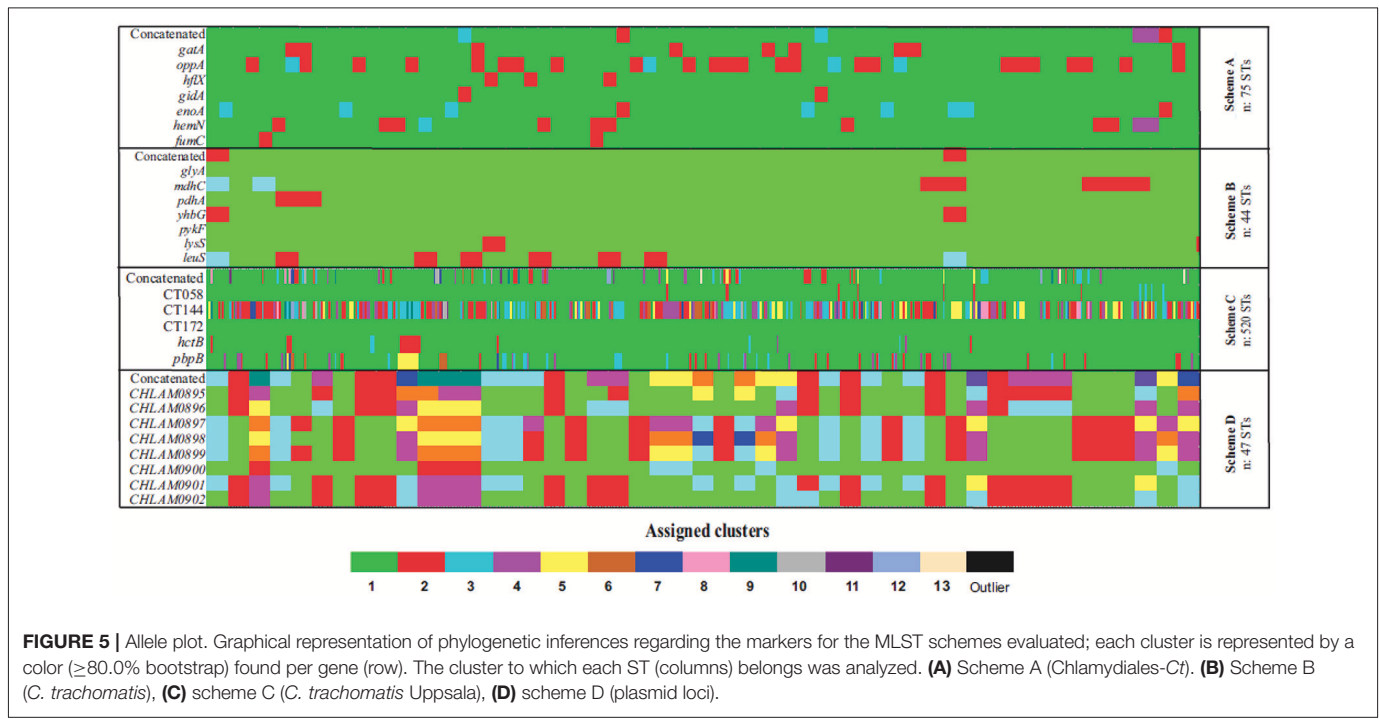
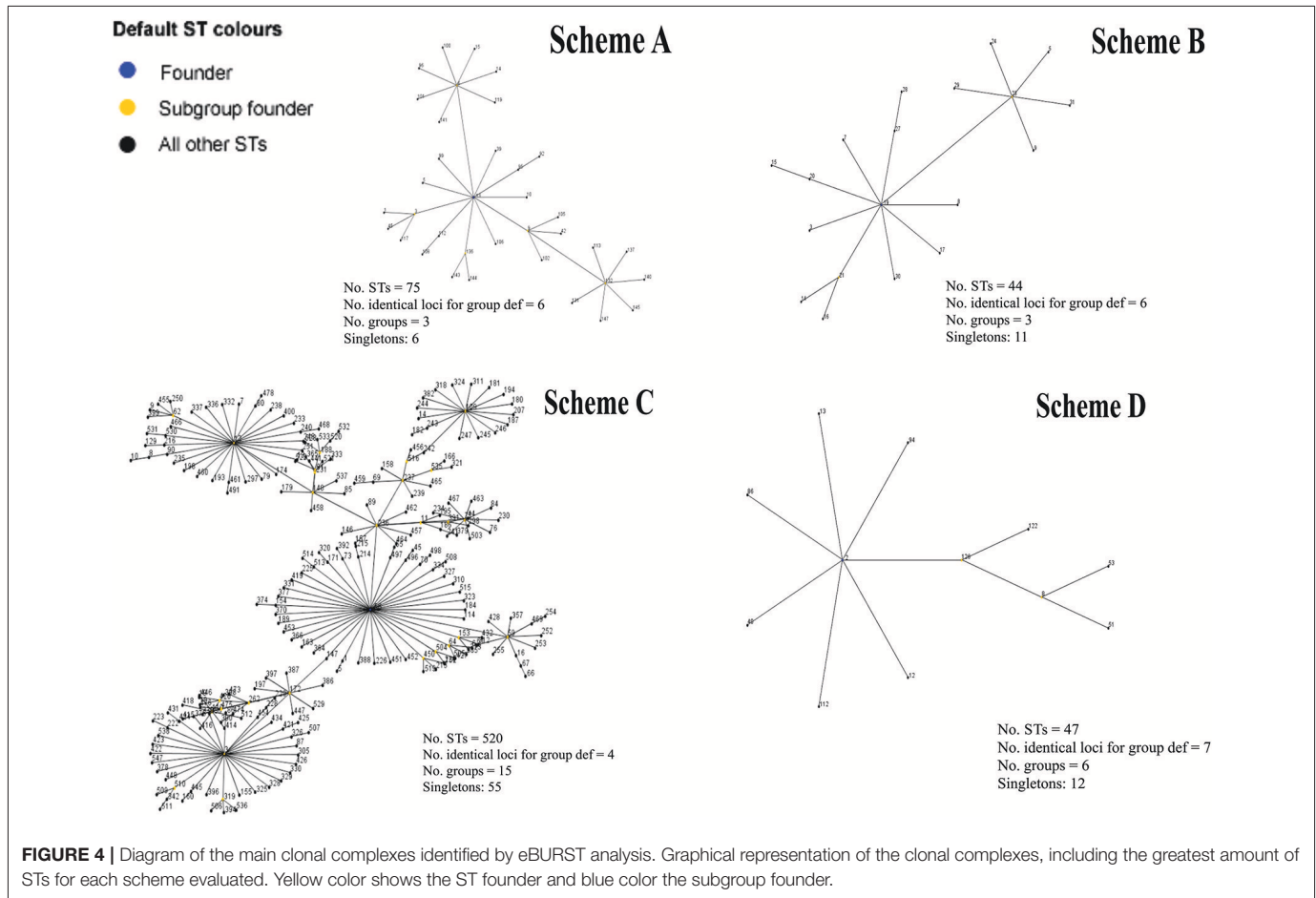
Finally, a set of 158 *Ct* genomes were subjected to ST identification, using the three MLST schemes targeting chromosomal genes. We identified 19 STs using Scheme A, 15 STs by Scheme B and 26 STs by Scheme C. Interestingly, allelic profiles and alleles that had not been reported in Chlamydiales MLST database were found within the data set, corresponding to 20 genomes for the case of Scheme A, 68 for Scheme B and 72 for Scheme C. The concatenated sequences for the seven house keeping genes of the Chlamydiales scheme were extracted and used to conduct multiple alignments and the subsequent phylogenetic reconstruction. The results showed reduced clustering discrimination by A and B schemes without evidence of clustering according to tropism (**Figures 6A,B**). In the case of Scheme C (**Figure 6C**),

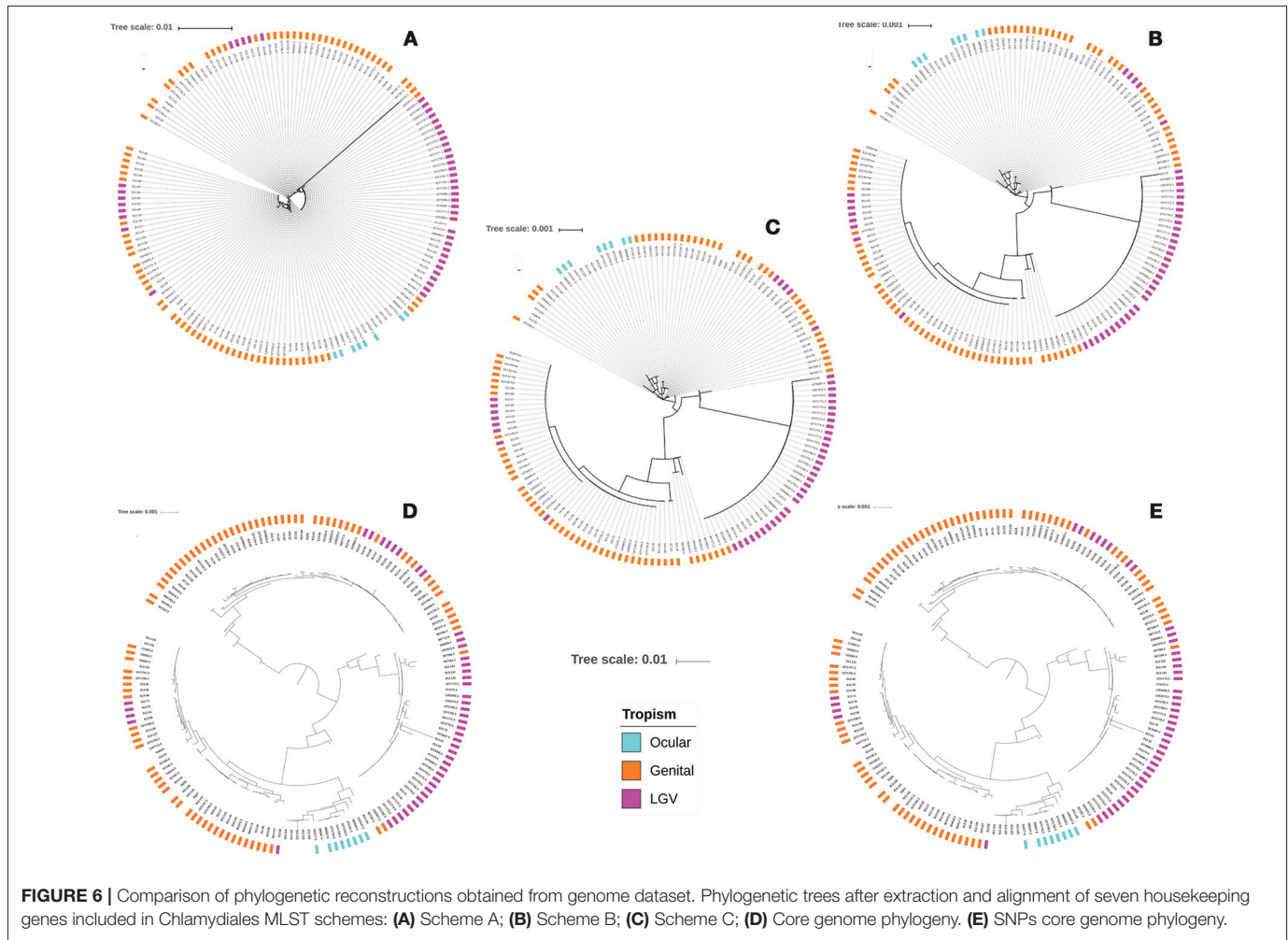
although clearer clustering profiles were identified, the topology of the tree did not allow a clear clustering by topology.

Considering that the core genome could be more informative in the evolutionary context. The annotated genomes (using prokka) were used to depict the *Ct* pangenome. A total of 3,177 genes defined the pangenome of the analyzed data set, of which 794 corresponded to the core genome. These genomes were used to perform the subsequent phylogenetic reconstructions (core genome phylogeny and core genome SNP phylogeny). When analyzing the data obtained in the core genome phylogeny, we observed the emergence of four clusters, whose sequences were mostly grouped according to their tropism (mainly those associated with ocular infections) (**Figure 6D**). This behavior was the same when performing the SNP core genome phylogeny (**Figure 6E**).

### Typing Efficiency and Discrimination Power for 27 Loci

Regarding typing efficiency (TE) (**Table 1**), the best results were obtained for Scheme B (1.0386 average) whose genes had >0.889 TE, except for *yhbG* (0.381). Analysis of Scheme A revealed increased TE for most genes (reaching an average of 0.7928); reduced TE was observed regarding *gidA* and *hemN*, the latter having the lowest value amongst the 27 markers evaluated here (0.026). The genes included in Scheme D had 0.7344 average TE; the gene having the greatest TE amongst the 27





molecular markers evaluated here was included in this scheme: *CHLAM0895* (1.500). Average ET for Scheme C was 0.3432, *CT058* being the marker giving the best result (0.750).

Evaluating discriminatory power (DP) (Table 1) revealed that Scheme C gave the highest results (0.8774 average) and included the genes having the highest result for all markers *hctb*: 0.925 and *pbpB*: 0.91. The Scheme D (0.7759 average) was the other scheme having high average DP; the markers giving the best results for these schemes were *CHLAM0898* (0.887) and *hctB* (0.925). Schemes A (0.5841) and B (0.5534) had the lowest DP. The marker having the lowest DP amongst the 27 genes was *mdhC* (0.289) from Scheme B.

Comparing TE and DP results for all markers revealed that only 8 markers had both results within the 75th percentile (Table 2). These markers' pattern was analyzed regarding the set of 179 isolates for which information was available for all MLST schemes. It was found that TE ranged from 0.883 (*leuS*) to 0.286 (*CHLAM0895*) and DP from 0.808 (*CT058*) to 0.603 (*leuS*).

After performing, a comparison with a "true scheme" as cgMLST and observing in general similar results with the compared schemes (MLST schemes herein evaluated). We decided to observe the plausibility of optimizing an

adequate MLST scheme with the available genes. Scheme optimisation revealed that the optimum number of loci required for identifying the largest amount of STs was 5; combinations of genes led to 69 STs being identified in this set of isolates. Even though 6 or more genes were included, a maximum of 72 STs were identified (Supplementary Table S6); *CHLAM902* gave the best TE and DP results for any of the last three markers (Table 2), meaning that this combination can be proposed as the optimum combination of markers for classifying this dataset. The harmonized proposal arises from analyses carried out here and is shown in Figure 7.

The concatenated sequences for all STs reported in each MLST scheme were used for the extraction of both SNPs and Pi sites, which were then analyzed for the usefulness of the MLST tool by determining the TE and DP (with their corresponding 95% CI), as described previously. These findings were compared with the results obtained from the complete sequences (Table 3), finding that the number of alleles identified for each MLST scheme from SNPs is almost equal to that of the complete sequence. Since, it is precisely these variations the characters informative by this approach, however the number

**TABLE 1** | Calculating the typing efficiency and discriminatory power of the markers in the schemes analyzed.

MLST scheme	Molecular marker	Typing efficiency	Mean per scheme [SD]	Discriminatory power [95% CI]	Mean per scheme [SD]	dN/dS
Scheme A	<i>gatA</i>	1.250	0.7928 [0.5305]	0.668 [0.586–0.751]	0.5841 [0.1741]	0.0094
	<i>oppA</i>	1.111		0.624 [0.516–0.732]		0.0191
	<i>htlX</i>	1.250		0.708 [0.634–0.782]		0.0184
	<i>gidA</i>	0.134		0.771 [0.717–0.826]		0.0200
	<i>enoA</i>	1.143		0.639 [0.56–0.718]		0.0184
	<i>hemN</i>	0.026		0.328 [0.191–0.464]		0.5880
	<i>fumC</i>	0.636		0.351 [0.211–0.492]		0.0172
	Combination of loci	0.148		1 [1–1]		
Scheme B	<i>glyA</i>	1.400	1.0386 [0.3401]	0.538 [0.372–0.705]	0.5534 [0.1918]	0.0096
	<i>mdhC</i>	1.333		0.289 [0.108–0.469]		0.0058
	<i>pdhA</i>	1.167		0.331 [0.139–0.523]		0.0109
	<i>yhbG</i>	0.381		0.526 [0.342–0.711]		0.0417
	<i>pykF</i>	1.000		0.739 [0.647–0.831]		0.0133
	<i>lysS</i>	0.889		0.668 [0.527–0.809]		0.0156
	<i>leuS</i>	1.100		0.783 [0.671–0.895]		0.0193
	Combination of loci	0.852		1 [1–1]		
Scheme C	CT058	0.75	0.3432 [0.2579]	0.875 [0.86–0.89]	0.8774 [0.0404]	0.0904
	CT144	0.277		0.829 [0.811–0.848]		0.2288
	CT172	0.092		0.848 [0.824–0.873]		0.6207
	<i>hctB</i>	0.178		0.925 [0.909–0.941]		0.0785
	<i>pbpB</i>	0.419		0.91 [0.903–0.918]		0.1429
	Combination of loci	0.183		1 [1–1]		
Scheme D	CHLAM0895	0.565	0.7344 [0.3559]	0.795 [0.708–0.881]	0.7759 [0.0252]	0.0251
	CHLAM0896	0.611		0.776 [0.675–0.878]		0.0181
	CHLAM0897	0.667		0.87 [0.826–0.913]		0.0133
	CHLAM0898	0.333		0.887 [0.841–0.933]		0.0201
	CHLAM0899	0.522		0.865 [0.819–0.91]		0.0289
	CHLAM0900	1.500		0.303 [0.131–0.476]		0.0075
	CHLAM0901	0.867		0.825 [0.759–0.891]		0.0189
	CHLAM0902	0.81		0.886 [0.837–0.935]		0.0282
	Combination of loci	0.586		1 [1–1]		

of alleles identified from Pi sites is reduced. For the case of TE and DP, the results are similar, in the case of Schemes B,C. The Pi sites show higher TE with respect to that determined from SNPs. In the case of Schemes A and D, the TE was reduced to almost half in the case of Pi sites, regarding the SNPs.

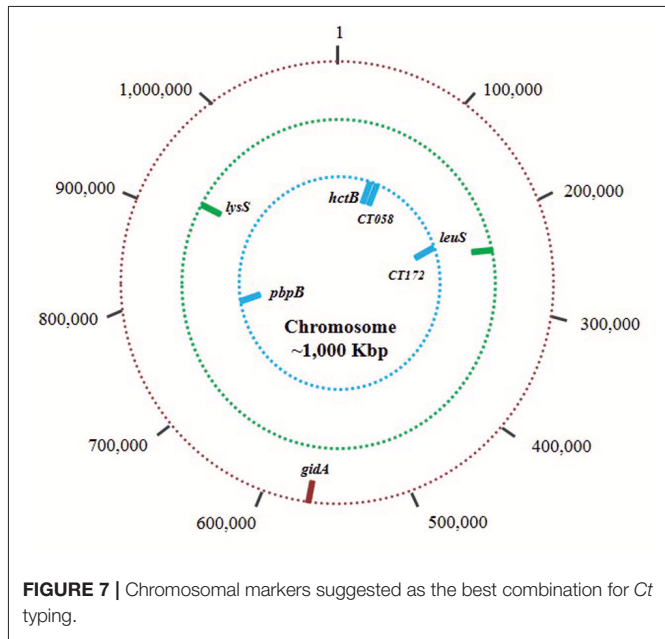
## DISCUSSION

Appropriate identification of *Ct* variants enables understanding infection's transmission dynamics and natural history. Different techniques have been developed for such purpose, including MLST, known for its high-resolution power and producing useful data for describing population structure at epidemiological, genetic and/or evolutionary levels (de Vries et al., 2015). There is a secure, open-access database for Chlamydiales (Chlamydiales MLST database <https://pubmlst.org/chlamydiales/>) which has information regarding the isolates obtained from different parts

**TABLE 2** | Typing efficiency and discriminatory power of the markers herein selected.

MLST scheme	Molecular marker	Typing efficiency	Discriminatory power (95% CI)
Scheme A	<i>gidA</i>	1.167	0.718 (0.684–0.751)
Scheme B	<i>leuS</i>	1	0.384 (0.299–0.468)
Scheme C	<i>LysS</i>	1	0.312 (0.227–0.398)
	CT058	0.517	0.823 (0.789–0.857)
	CT172	0.056	0.879 (0.849–0.908)
	<i>hctB</i>	0.052	0.843 (0.803–0.883)
	<i>pbpB</i>	0.194	0.874 (0.857–0.891)
Combination of loci		0.139	0.976 (0.969–0.984)

of the world and also contains sequences from different schemes, including the four MLST schemes (Jolley and Maiden, 2010; Maiden et al., 2013). The different typing schemes evaluated have been developed for different purposes (analysis of evolutionary



changes, discrimination between strains and epidemiological analyzes). However, it is necessary to find a limited number of MLST markers that provide the best discriminatory power that can subsequently be employed within a single, effective and efficient scheme that can be used globally.

The results obtained in these databases allowed us to conduct a descriptive analysis of the information contained therein and although the original data is not population based, allowed us to compare them with what was currently reported in the literature. One of these analyzes allowed us to determine that variants E, D, F, and G (included in Cluster 2) occurred most frequently regarding the 19 variants currently known for *Ct* (Figures 2A–D); this agreed with that reported previously, where close to 50% of genital tract infections caused by *Ct* were associated with such variants (Nunes et al., 2010; O'connell and Ferone, 2016). On the other hand, it has been observed that *Ct* variants are grouped according to three types of pathology and tissue tropism (ocular, urogenital and LGV). However and interestingly, the results obtained from the database indicated that variants such as those belonging to Cluster 1 (reported as being related with ocular tropism) had been exclusively isolated from genitourinary samples, which can partly explain why *Ct* variants were able to colonize differing ecological niches (Harris et al., 2012) (Ferreira et al., 2014). Finally, we observed that around 95% of the isolates included in the database were from individuals aged 16–29 years-old, thereby agreeing with the information reported by the Centers for Disease Control and Prevention, which has described that most cases associated with *Chlamydia* are presented in adolescents and young adults (Lagkouvardos et al., 2014; O'connell and Ferone, 2016). The currently available epidemiological and clinical data has revealed high *Ct* infection prevalence worldwide; such information has led to the development of screening and molecular typing methods for evaluating the impact of infection by Chlamydiales species and broadening knowledge concerning

its genetic and population structure (Gharsallah et al., 2016; Versteeg et al., 2016). Analyzing the four MLST schemes available for *Ct* has led to identifying characteristics related to inter- and intra-taxa discrimination power.

Regarding Scheme A (based on housekeeping genes and directed toward inter-species identification), the results showed that all its genes had a low percentage of identity and nucleotide diversity index (Figure 3), this being the only scheme where it was observed that only one marker (*hemN*) had a large number of informative sites (>0.9) (Supplementary Figure S4). Concerning population structure analysis, a short amount of CCs was observed ( $n = 3$ ) (Figure 4), this result was confirmed by the low number of clusters produced in the allele plot (Figure 5) and few reticulation events in phylogenetic networks (Supplementary Figure S5). The results suggested that their use in intra-species discrimination would be debatable.

Regarding intra-species identification, Scheme B (also based on housekeeping genes), it was found that all the genes had a high percentage of identity, reduced number of informative sites (Supplementary Figure S4) and low nucleotide diversity indices, thereby indicating their conserved nature (Figure 3). Concerning population structure, it was found that 3 CCs grouped most STs reported here ( $n = 33/44$ ) (Figure 4), this being confirmed by the uniformity regarding STs discrimination, described by the reduced number of clusters in the allele plot (Figure 5) and the limited amount of reticulation events in the phylogenetic networks (Supplementary Figure S5). These results would thus suggest that the genes in this scheme did not have sufficient discrimination and/or typing power for intra-taxa classification. This could have been related to the high degree of genome conservation between *Ct* serovarieties (~99%) (Ferreira et al., 2012), meaning that the use of this scheme should be re-evaluated. These findings were similar to those for Scheme A (also based on housekeeping genes), that showed a low discriminatory power and seem not to be quite useful for evaluating intra-taxa diversity.

Regarding Scheme C (based on hypervariable genes), intra-taxa analysis identified that most genes in this scheme had heterogeneous percentages of identity and amount of variable and informative sites (Supplementary Figure S4), accompanied by reduced diversity indices (except for the CT172 gene) (Figure 3). Regarding population structure, this scheme produced most CCs ( $n = 15$ ) (Figure 4), showing a high degree of intra-taxa diversity, corroborated by the large number of clusters in the allele plot (except for the C172 gene) (Figure 5) and by the high degree of reticulation in the phylogenetic networks (Supplementary Figure S5). Using schemes exclusively including hypervariable genes could thus overestimate diversity in terms of population structure, as has been observed in other pathogens, such as *Candida albicans* (McManus and Coleman, 2014).

Regarding scheme D, the results showed that all genes in such scheme had high identity (Figure 3 and Supplementary Figure S4), few variable sites and heterogeneity concerning the number of informative sites, including totally informative markers, such as CHLAM0895 and CHLAM0898 (Supplementary Figure S4), as well as low nucleotide diversity,

**TABLE 3** | Calculation of SNPs and Pi sites according to the scheme evaluated.

	Scheme A			Scheme B			Scheme C			Scheme D		
	Complete sequence	SNPs	Pi sites	Complete sequence	SNPs	Pi sites	Complete sequence	SNPs	Pi sites	Complete sequence	SNPs	Pi sites
Number of alleles	75	74	53	44	44	37	520	501	454	47	46	16
Number of polymorphisms	435	223	198	61	61	45	1747	665	520	161	140	93
Typing efficiency	0.172	0.332	0.268	0.721	0.721	0.822	0.298	0.753	0.873	0.292	0.329	0.172
DP (95% confidence interval)	1 (1–1)	1 (0.999–1)	0.986 (0.977–0.995)	1 (1–1)	1 (1–1)	0.99 (0.98–1)	1 (1–1)	1 (1–1)	0.999 (0.999–1)	1 (1–1)	0.999 (0.996–1)	0.913 (0.877–0.949)

thereby agreeing with the conserved nature previously reported for these elements. Concerning population structure, 6 CC were identified (Figure 4), showing moderate intra-taxa diversity, supported by the number of clusters observed in the allele plot. The CHLAMY0895 gene had high discrimination power (8 clusters) (Figure 5), possibly being an indicator of recombination events. However, the discrete reticulation found in the phylogenetic network (Supplementary Figure S5) suggested that intra-taxa diversity may not have been suitably identified as it has been identified by other markers, such as *OmpA*, where it has been observed that true diversity has been masked (Harris et al., 2012). Plasmid loci's informative capability could be related to mobile genetic elements or errors during transduction events enabling the emergence of molecular rearrangements, ultimately affecting bacterial fitness (Sigar et al., 2014). Recent studies have shown that the presence of these plasmids governs chromosomal gene transcription related to the pathogenic effect, thereby being proposed as virulence factors for this species (Zhong, 2017).

In the absence of a “true scheme” that could help us to determine the best scheme for understanding the *Ct* molecular epidemiology. We decided to retrieve the available *Ct* genomes and compare the cgMLST and SNP cgMLST phylogenies with the available MLST schemes (A–D).

Initially, the results obtained with the 16S rRNA phylogeny showed the scarce utility of this marker for the intra-species typing of *Ct*, due to its limited discrimination and genotyping power (Supplementary Figure S6) and its restricted intra-species classification capacity (presents limited informative sites), mainly in the classification between organisms with closely related genomes (Cooper and Feil, 2004; Carrasco et al., 2013). Regarding phylogenies inferred using core genome and SNPs-core genome, they showed similar topology and clustering according to tropism. The results also confirm that phylogenetic approaches that start from complete genomes, provide a greater discriminatory power at the intra-species level (Figure 6) (Versteeg et al., 2018); typing schemes aimed at the core genome allow to detect minimum changes at the genome level between variants, allowing a more robust classification. This methodology is advantageous and presents better resolution to those schemes based on MLST, mainly in microorganisms with highly conserved genomes (Gonzalez-Escalona et al., 2017; Tsang et al., 2017; Versteeg et al., 2018). However, in general the clustering (using

genomic data) did not significantly differ from that based on MLST schemes herein evaluated. In terms of feasibility, we conclude that MLST might be more accessible and hypothesis driven than cgMLST.

Despite the schemes evaluated have been developed for different purposes (schemes A,B have been suitable for evolutionary studies and the scheme C for short-term clinical epidemiology and outbreak investigations) and have been based on different targets (housekeeping and hypervariable genes), which can generate a bias in the analyses obtained. They represent the only information currently available worldwide for genotyping *Chlamydiales*. The findings show that the four MLST schemes available for *Ct* described to date do not have suitable behavior for describing circulating genotypes and thus cannot adequately describe inter- and intra-taxa diversity. However, analysis of individual markers showed compliance with the criteria required for being used in an MLST scheme, i.e., suitable typing efficiency, high discrimination power and a lack of stabilizing selective pressure (dN/dS lower than 1.0) (Table 1). Analyzing the optimization of the scheme using the set of markers having the best behavior (Table 2) led to suggesting the use of 3 loci, currently included in B (housekeeping gene), C (hyper-variable gene), and D MLST schemes (plasmid loci), as being the best combination of genes for *Ct* typing (Figure 7). They had optimized typing efficiency concerning the dataset evaluated here and also maintained maximum discrimination power. Even though some isolates lose plasmids (Sigar et al., 2014), their important role regarding the impact of *Ct* on a particular host ratifies their usefulness as typing marker, meaning that even their absence should be considered within a scheme applicable to clinical isolates.

However, in circumstances where access to the core genome is restricted, either by the availability of information, samples or by computational tools (Yi and Jin, 2013; Taylor-Brown et al., 2016), the MLST could be considered as a good tool for intra-species typing because it has been shown to be reproducible, highly discriminatory and easy to implement in the laboratory (Cooper and Feil, 2004). Although its use can lead to analysis of small-scale evolutionary changes, given the use of only one set of molecular markers (Tsang et al., 2017). Herein, we show MLST optimization as an alternative for *Ct* typing that showed to be in overall compatible with

the cgMLST and SNP cgMLST. It is well known that the WGS is the best tool to assess the variability and to improve the understanding of inter- and intraspecies phylogenetic relationships (Tsang et al., 2017); however, this requires a more complex infrastructure, as its higher costs and the analysis of the data is more complex. With this article we intend to generate a cost-effective tool that allow an identification of circulating strains in short time, which in the future may contribute to characterizing outbreak transmission, monitoring relapses (recurrence/reinfection) (de Vries et al., 2015), and identifying the genetic variability of species infecting multiple hosts. Taken together, the above will contribute toward the surveillance of emergent genotypes and understanding the genetic causes of the disease's physiopathological mechanisms.

## AUTHOR CONTRIBUTIONS

LP, MC, MM and JR conceived and designed the study, analyzed and interpreted the data and prepared the manuscript. DR-C, JR and MP critically read the manuscript and made important suggestions. JR conceived and designed the study and revised

the manuscript. All authors have reviewed and approved the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2018.01854/full#supplementary-material>

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# The effect of six types of high-risk human papillomavirus viral load on Chlamydia trachomatis infection, clearance and persistence

Milena Camargo<sup>1</sup>, Ricardo Sánchez<sup>2</sup>, Indira M. Báez-Murcia<sup>1</sup>, Sara C. Soto-De León<sup>1</sup>, Manuel E. Patarroyo<sup>1</sup>, Manuel Alfonso A. Patarroyo<sup>1, 3\*</sup>

<sup>1</sup>Colombian Institute of Immunology Foundation, Colombia, <sup>2</sup>National University of Colombia, Colombia, <sup>3</sup>Rosario University, Colombia

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***CAPÍTULO 2***

***COMPONENTE TRANSVERSAL***

RESEARCH ARTICLE

Open Access

# Human papillomavirus detection in women with and without human immunodeficiency virus infection in Colombia

Milena Camargo<sup>1,2</sup>, Sara C Soto-De Leon<sup>1,2</sup>, Marina Munoz<sup>1,2</sup>, Ricardo Sanchez<sup>1,3</sup>, Diego Peña-Herrera<sup>4</sup>, Andrea Clemencia Pineda-Peña<sup>5</sup>, Otto Sussmann<sup>6</sup>, Carol Paez<sup>6</sup>, Antonio Perez-Prados<sup>7</sup>, Manuel Elkin Patarroyo<sup>1,3</sup> and Manuel Alfonso Patarroyo<sup>1,2\*</sup>

## Abstract

**Background:** HIV infection leads to a decreasing immune response, thereby facilitating the appearance of other infections, one of the most important ones being HPV. However, studies are needed for determining associations between immunodeficiency caused by HIV and/or the presence of HPV during the course of cervical lesions and their degree of malignancy. This study describes the cytological findings revealed by the Papanicolaou test, laboratory characteristics and HPV molecular profile in women with and without HIV infection.

**Methods:** A total of 216 HIV-positive and 1,159 HIV-negative women were invited to participate in the study; PCR was used for the molecular detection of HPV in cervical samples. Statistical analysis (such as percentages, Chi-square test and Fisher's exact test when applicable) determined human papillomavirus (HPV) infection frequency (single and multiple) and the distribution of six types of high-risk-HPV in women with and without HIV infection. Likewise, a logistic regression model was run to evaluate the relationship between HIV-HPV infection and different risk factors.

**Results:** An association was found between the frequency of HPV infection and infection involving 2 or more HPV types (also known as multiple HPV infection) in HIV-positive women (69.0% and 54.2%, respectively); such frequency was greater than that found in HIV-negative women (44.3% and 22.7%, respectively). Statistically significant differences were observed between both groups ( $p = 0.001$ ) regarding HPV presence (both in infection and multiple HPV infection). HPV-16 was the most prevalent type in the population being studied ( $p = 0.001$ ); other viral types had variable distribution in both groups (HIV-positive and HIV-negative). HPV detection was associated with  $<500 \text{ cell/mm}^3$  CD4-count ( $p = 0.004$ ) and higher HIV-viral-load ( $p = 0.001$ ). HPV-DNA detection,  $<200 \text{ cell/mm}^3$  CD4-count ( $p = 0.001$ ), and higher HIV-viral-load ( $p = 0.001$ ) were associated with abnormal cytological findings.

**Conclusions:** The HIV-1 positive population in this study had high multiple HPV infection prevalence. The results for this population group also suggested a greater association between HPV-DNA presence and cytological findings. HPV detection, together with low CD4 count, could represent useful tools for identifying HIV-positive women at risk of developing cervical lesions.

**Keywords:** Human papillomavirus, Human immunodeficiency virus, Multiple infection, Papanicolaou test, Epidemiology

\* Correspondence: [mapatarr.fidic@gmail.com](mailto:mapatarr.fidic@gmail.com)

<sup>1</sup>Fundación Instituto de Inmunología de Colombia, Cra. 50 # 26-20, Bogotá, Colombia

<sup>2</sup>School of Medicine and Health Sciences, Universidad del Rosario, Calle 14 # 6 - 25, Bogotá, Colombia

Full list of author information is available at the end of the article

## Background

Epidemiological and molecular studies have led to a causal relationship being established between infection involving certain types of human papillomavirus (HPV), known as high risk (HR-HPV), and the development of cervical cancer (CC) [1]. Fifteen of the viral types identified to date have been classified as HR-HPV (HR-HPV-16, -18, -45, -31, -33, -52, -58 and -35 having the highest frequency) [1].

This type of cancer accounts for the second cause of death by malignant neoplasia in women, primarily those of childbearing age and living in developing countries. A CC mortality rate of 18.2 for every 100,000 women per year was reported in Colombia in 2010 [2].

HPV infection (considered a common sexually-transmitted infection) is necessary but not the sole factor for CC to develop; several risk factors can trigger the development of this cancer, immunosuppression being one of the most significant ones [3-5].

One of the most studied types of immunosuppression is that due to human immunodeficiency virus (HIV) infection [6]. Such infection leads to alterations in cell-mediated immunity, thereby facilitating the acquisition of opportunistic infections and limiting an organism's ability to produce an efficient immune response [7].

Higher HPV infection prevalence has been documented in HIV-infected women; the foregoing, taken together with a deficient immune system, thus contributes towards new infections involving other HPV types being acquired (multiple infection or the coexistence of 2 or more HPV types) which, as the virus cannot be eliminated, increases HPV infection persistence in the body [5,8].

The clinical significance of HPV persistence concerns this virus' long-term existence. This allows for the efficient replication and integration of viral oncoproteins (E6 and E7) in the host genome, mutations to become accumulated, genomic instability and chromosomal aberrations; this causes rapid morphological changes to cells, thereby contributing towards CC development [9,10].

Mild dysplasia prevalence in immunocompetent women does not exceed 27%, whereas this can rise to 80% in HIV-positive women; immunosuppression in the latter group implies the development of more aggressive lesions and a lower response to treatment [11], as well as leading to the appearance of precancerous lesions in about 60% of women suffering HPV infection which evolves in less than 3 years [11,12].

It has been described that HIV-positive women having a CD4 count of less than 200 cells/mm<sup>3</sup> have greater HPV infection prevalence and increased cervical intraepithelial lesion incidence rates; the foregoing, together with a high HIV viral load, significantly increases the risk of developing this type of lesion [13].

It has been stated that the relationship between HIV-HPV might become a worldwide public health problem [14]; however, some reports have shown a low correlation between HIV infection and the development of neoplastic lesions. Such correlation mainly occurs in low-income countries having limited access to antiretroviral therapy (ART) [10,15].

No studies have been carried out to date in Colombia to determine HIV-HPV infection-related epidemiological profiles, so this study was aimed at describing the prevalence of multiple HPV infection, involving the type-specific distribution of six HR-HPV types amongst women with and without HIV infection involved in sexual and reproductive health programmes at four hospitals in Bogota. The resulting information has contributed towards knowledge regarding HIV-HPV-related sexually-transmitted infections.

## Methods

### Study design

This was a cross-sectional study; the patients participated between February 2007 and November 2012. All hospitals included in this study are located in the country's capital city, Bogotá. The group of HIV-positive women (classified as having human immunodeficiency virus type 1) were attending a programme being run by Asistencia Científica de Alta Complejidad S.A.S (n = 216). The HIV-negative women were participating in sexual and reproductive health programmes being provided by state-run healthcare institutes; this group of women was recruited at the Hospital de Bosa (n = 232), Hospital de Fontibón (n = 290) and the Hospital de Engativá (n = 637), all located in Bogotá. The participating institutions reported the HIV status for all the women included in the study.

All the women were told about the study's purpose. Those who volunteered to participate signed an informed consent form and completed a questionnaire aimed at collecting data concerning socio-demographic characteristics and risk factors before being given a gynaecological exam. An informed consent form, signed and approved in the presence of a parent or guardian, was required for women younger than 18 years old. This study was approved and supervised by all health care participating institutions' Ethics Committees as follows: the Bioethics Committee of 'Asistencia Científica de Alta Complejidad', the Ethics Committee of the 'Hospital de Bosa E.S.E.', the Ethics Committee of the 'Hospital de Fontibón E.S.E.', and the Hospitalary Ethics Committee of the 'Hospital de Engativá Nivel II'.

The inclusion criteria took into account the HIV status reported by the participating institutions, the women's voluntary participation in this study by signing the consent form and filling in a questionnaire which considered socio-demographic characteristics and risk factors.

Exclusion criteria considered women where a  $\beta$ -globin gene segment could not be amplified.

#### Sample collection and methodological design

Papanicolaou test (Pap test) samples were collected following Colombian Ministry of Health guidelines for the early detection of CC [16]; the Pap smears were read by each participating hospital. Cytological findings were reported according to the Bethesda system as being atypical squamous cells of undetermined significance (ASCUS), a low grade squamous intraepithelial lesion (LSIL) or a high grade squamous intraepithelial lesion (HSIL).

Cervical samples for detecting HPV-DNA were obtained during cytological exam; cells were collected from the cervix using a cytobrush which was stored in a tube containing 95% ethanol as a means of preservation and transport [17].

The methodological design for detecting HPV involved using PCR, firstly, directed towards determining DNA quality and integrity (using primers directed towards detecting the  $\beta$ -globin gene segment). Independent PCRs were then used for the generic detection of HPV, using three sets of primers directed towards detecting segments encoding viral proteins L1 and E6-E7. Independent PCRs were also used on samples where generic detection had revealed the virus; type-specific identification involved using primers directed towards genes encoding early proteins E5, E6 and E7 [17,18].

#### Sample processing and human papillomavirus DNA detection

Total DNA was extracted from cervical cells using a commercial QuickExtract solution kit (Epicentre, Madison, WI), following the manufacturer's instructions.

PCR analysis involved GH20/PC04 and PC03/PC04 specific primers for detecting a  $\beta$ -globin gene segment in independent PCR reactions to confirm the presence of human DNA in all the samples [17].

Established PCR techniques were used for HPV-DNA detection [17,18]. Viral genes were detected using three primer sets; pU1M/2R was directed to the region encoding virus oncogenic proteins (E6/E7) and GP5+/6+ and MY09/11 directed towards a segment encoding the L1 late protein [17].

Samples having a positive result for HPV-DNA by PCR (samples proving positive for one or more of the three generic primer sets) were used for type-specific identification using primers targeting regions encoding viral oncogenic proteins E5, E6 or E7 from six high-risk HPV types (HR-HPV-16, -18, -31, -33, -45, -58), these being the most prevalent in the Colombian population and accounting for 90% of CC cases [17].

All PCRs were run simultaneously in separate reactions and in previously described conditions [17,18].

#### Statistical analysis

Sample size was determined as being 179 in the HIV-positive group and 716 for the HIV-negative group to ensure 80% power for detecting a 1.60 odds ratio (OR). The proportion in group one (HIV-positive women) was assumed to be 0.45 by null hypothesis and 0.55 by alternative hypothesis.

A descriptive analysis of the demographic characteristics and risk factors was made and they were treated as categorical variables (mean, standard deviation (SD), percentages); estimations were made, along with their respective 95% confidence intervals (95% CI). Chi-square and Fisher's exact tests were used (when applicable) to evaluate differences in proportions. The coexistence of 2 or more HPV types was defined as multiple infection in this study. The strength of association was measured using ORs with 95% CI; logistic regression was used for ordinal data to estimate adjusted ORs. The cofactors included in regression analysis were age, marital status, age at first intercourse, number of pregnancies, having had other STDs, the number of lifetime sexual partners, contraceptive methods used and smoking status, along with laboratory characteristics (Papanicolaou test, CD4-count, HIV-viral-load and ART). All hypothesis tests were set at 0.05 significance level. STATA11 was used for all statistical procedures.

#### Results

The study involved 1,375 women aged 14–76 years (SD = 10.9; mean age = 36.9: 36.3-37.5 95% CI). The predominant ethnic group consisted of 1,337 mestizo women (97.2%: 96.2-98.1 95% CI), the remaining 38 (2.8%: 1.92-3.65 95% CI) being Afro-descendants and indigenous females.

216 of the women were HIV-positive whose ages ranged from 20–73 years old (SD = 10.7; mean age = 37.5: 36.0-38.9 95% CI) and a second group consisted of 1,159 HIV-negative women aged 14–76 (SD = 11.0; mean age = 36.8: 36.1-37.4 95% CI). The estimator revealed statistically significant differences regarding some women's socio-demographic characteristics and risk factors according to HIV status (such as ethnicity and age at first intercourse), whilst no statistically significant difference was recorded for pregnancies ( $p = 0.173$ ) and/or infection with other sexually transmitted diseases ( $p = 0.071$ ) (Table 1).

All samples ( $n = 1,375$ ) which amplified for the  $\beta$ -globin gene and tested for HPV-DNA presence (indicated by positive amplification of pU1M/2R, GP5+/6+ or MY09/11, or more than one) were used in the statistical analysis. The virus was detected in 48.1% of the samples ( $n = 662$ : 45.5-50.8 95% CI) and multiple infection (defined as coexistence of 2 or more HPV types) in 57.4% ( $n = 380$ : 53.5-61.7 95% CI) of HPV positive samples.

**Table 1 Demographic profile of the 1,375 women having positive human  $\beta$ -globin amplification**

Variable	HIV-positive		HIV-negative		p value
	(n = 216)		(n = 1,159)		
<b>Age*</b>	37.5 [20–73] SD = 10.7		36.8 [14–76] SD = 11.0		
<b>Ethnicity</b>	<b>n</b>	<b>(%)</b>	<b>n</b>	<b>(%)</b>	
Indigenous	3	(1.4)	2	(0.2)	
Mestizo	209	(96.8)	1,128	(97.3)	
Afro-descendant	4	(1.8)	29	(2.5)	0.021
<b>Marital status</b>					
Single	66	(30.6)	301	(26.0)	
Married	36	(16.7)	202	(17.4)	
Cohabiting	65	(30.1)	497	(42.9)	
Separated	22	(10.2)	132	(11.4)	
Widowed	27	(12.4)	27	(2.3)	0.001
<b>Age at first intercourse</b>					
<15	75	(34.7)	276	(23.8)	
16–19	111	(51.4)	618	(53.3)	
>19	30	(13.9)	265	(22.9)	0.001
<b>Pregnancies</b>					
None	10	(4.6)	69	(6.0)	
1–3	160	(74.1)	784	(67.6)	
>4	46	(21.3)	306	(26.4)	0.173
<b>Abortions</b>					
No	120	(55.7)	741	(64.0)	
Yes	96	(44.3)	418	(36.0)	0.019
<b>Other STD</b>					
No	136	(63.0)	653	(56.4)	
Yes	80	(37.0)	506	(43.6)	0.071
<b>Lifetime number of sexual partners</b>					
1–2	88	(40.7)	783	(67.5)	
>3	128	(59.3)	376	(32.5)	0.001
<b>Contraceptive method</b>					
None	43	(19.9)	337	(29.1)	
Hormonal	11	(5.1)	189	(16.3)	
Surgery	46	(21.3)	318	(27.4)	
Condom	97	(45.0)	113	(9.8)	
Intrauterine device	19	(8.7)	202	(17.4)	0.001
<b>Smoking status</b>					
No	177	(81.9)	776	(66.9)	
Yes	39	(18.1)	383	(33.1)	0.001
<b>Cytological findings</b>					
Normal	148	(68.5)	918	(79.2)	
Abnormal	68	(31.5)	241	(20.8)	0.006

p value: Probability value, SD: standard deviation, STD: sexually transmitted diseases.

\*Mean [range]; SD.

HPV-DNA was detected in 44.3% of samples from HIV-negative women (n = 513: 41.4-47.2 95% CI) and multiple infection in 51.3% of them (n = 263: 46.8-55.7 95% CI). The presence of HPV was observed in 69.0% of HIV-positive women (n = 149: 62.3-75.0 95% CI) and multiple infection was found in 78.5% of the sample (n = 117: 71.0-84.8 95% CI). Statistically significant differences were observed between both groups of women (p = 0.001) regarding HPV presence (both in infection and multiple HPV infection).

Cytological findings proved negative for intraepithelial lesions or malignancy in 68.5% of the HIV-positive women (n = 148: 61.9-74.7 95% CI) and abnormality occurred in 31.5% of them (n = 68: 25.3-38.1 95% CI), classified as follows: ASCUS occurred in 35.3% of cytological abnormalities (n = 24: 24.1-47.8 95% CI), LSIL in 57.3% (n = 39: 44.8-69.3 95% CI) and HSIL in 7.4% of them (n = 5: 2.4-16.3 95% CI).

79.2% (n = 918: 76.7-81.5 95% CI) of HIV-negative women proved negative for lesions, whilst 20.8% of them (n = 241: 18.5-23.2 95% CI) had some degree of abnormality: 52.7% (n = 127: 46.2-59.1 95% CI) had ASCUS, 41.5% (n = 100: 35.2-47.9 95% CI) had LSIL and 5.8% (n = 14: 3.21-9.55 95% CI) HSIL. The difference between abnormality detected by Pap test for women having different HIV infection status was statistically significant (p = 0.003).

Association between HPV status and Pap test result determined by ORs revealed a positive trend for HPV-DNA detection and ASCUS cytological findings (crude OR 1.85: 1.31-2.63 95% CI). A positive association was observed when comparing SIL cytological findings (crude OR 1.70: 1.21-2.39 95% CI; test of trends in odds:  $\text{Chi}^2(1) = 16.13$  p = 0.001). Calculating regression analysis association between HIV status and Pap test result revealed a positive trend for HIV-positive and SIL (crude OR 2.39: 1.61-3.54 95% CI; test of trends in odds:  $\text{Chi}^2(1) = 18.04$  p = 0.001). All associations remained statistically significant after using logistical regression to adjust for cofactors (described in the statistical analysis section) (Table 2).

Positive associations (using regression analysis) between abnormal cytological findings (including ASCUS, LSIL and HSIL) and clinical and laboratory characteristics (CD4 count, HIV viral load and antiretroviral therapy-ART) showed a positive trend for CD4 cell count below 200 cell/mm<sup>3</sup> (crude OR 2.96: 1.85-4.73 95% CI; test of trends in odds:  $\text{Chi}^2(1) = 23.03$  p = 0.001), higher than 100,000 copies/mL HIV viral load (crude OR 10.47: 3.26-33.60 95% CI; test of trends in odds:  $\text{Chi}^2(1) = 23.40$  p = 0.001) and women with and without ART treatment (crude OR 1.57: 1.11-2.21 95% CI, and crude OR 4.19: 1.75-10.03 95% CI; test of trends in odds:  $\text{Chi}^2(1) = 15.96$  p = 0.001) (Table 3).

ORs were used for assessing the association between women with and without HIV infection and having HPV (prevalence, infection status, clade detected and clinical and laboratory characteristics). The results revealed statistically significant associations between HIV-positive women and an increased likelihood of HPV-DNA detection (crude OR 2.80: 2.04-3.84 95% CI; p = 0.001), multiple infection (crude OR 4.02: 2.94-5.49 95% CI; p = 0.001), clade 7 (crude OR 2.62: 1.90-3.62 95% CI; p = 0.001) and clade 9 detection (crude OR 2.91: 2.14-3.97 95% CI; p = 0.001), lower than 500 cell/mm<sup>3</sup> CD4 cell count (crude OR 2.02: 1.11-3.70 95% CI; p = 0.004), higher than 100,000 copies/mL HIV viral load (crude OR 8.18: 1.82-36.71 95% CI; p = 0.001) and women with and without ART treatment (crude OR 2.68: 1.93-3.72 95% CI; p = 0.001 and crude OR 3.52: 1.25-9.88 95% CI; p = 0.005). These associations remained significant in multivariate analysis (Table 4).

Regarding type-specific distribution in HIV-positive and negative groups, HPV-16 was the most prevalent infection for the entire study population (p = 0.001). Significant differences were observed regarding the percentages of HPV infection types for each population, HPV-31 was the second most prevalent in HIV-positive women (p = 0.001), HPV-18 the third (p = 0.001) (Table 5) whilst HPV-58 was the second most prevalent in HIV-negative women (p = 0.215) followed by HPV-31 (p = 0.001). HPV-45 occurred least frequently in both populations (p = 0.001) (Table 5).

**Table 2 Multivariate analysis of HPV status and HIV status in women regarding cytological findings**

Pap test result	HPV status					HIV status				
	Negative		Positive		Adjusted OR (95% CI)	Negative		Positive		Adjusted OR (95% CI)
	n	(%)	n	(%)		n	(%)	n	(%)	
Negative	587	(82.3)	479	(72.4)	Ref	918	(79.2)	148	(68.5)	Ref
ASCUS	60	(8.4)	91	(13.7)	<b>1.84</b> <b>1.28-2.62</b>	127	(11.0)	24	(11.1)	1.18 0.70-1.99
SIL*	66	(9.3)	92	(13.9)	<b>1.69</b> <b>1.19-2.41</b>	114	(9.8)	44	(20.4)	<b>2.74</b> <b>1.74-4.33</b>
Total	713		662			1,159		216		

OR adjusted for age, marital status, age at first intercourse, the number of lifetime sexual partners, contraceptive methods used and smoking status, Papanicolaou test, CD4-count, HIV-viral-load and antiretroviral therapy or ART.

95% CI: confidence interval, OR: odds ratio, ASCUS: atypical squamous cells of undetermined significance, SIL: squamous intraepithelial lesions.

Values in bold = p < 0.05.

\*SIL include: low grade squamous intraepithelial lesion (LSIL) and high grade squamous intraepithelial lesions (HSIL).

**Table 3 Multivariate analysis of factors associated with abnormal cytological findings**

	Abnormal Pap test result *			
	n	(%)**	Adjusted OR	95% CI
<b>HIV; immune status CD4 cell/mm<sup>3</sup> count</b>				
HIV-negative	241	(20.8)	Ref	
HIV-positive, < 200	35	(16.2)	<b>2.89</b>	<b>1.73-4.84</b>
HIV-positive, 200 - 349	12	(5.6)	1.05	0.52-2.11
HIV-positive, 350 - 500	12	(5.6)	1.34	0.66-2.71
HIV-positive, > 500	9	(4.2)	1.41	0.63-3.16
<b>HIV viral load copies/mL</b>				
HIV-negative	241	(20.8)	Ref	
HIV-positive, <4,000	49	(22.7)	1.44	0.96-2.16
HIV-positive, 4,000-99,999	8	(3.7)	1.95	0.77-4.94
HIV-positive, >100,000	11	(5.1)	<b>8.63</b>	<b>2.65-28.12</b>
<b>HIV and ART use</b>				
HIV-negative	241	(20.8)	Ref	
HIV-positive, treatment with ART	57	(26.4)	<b>1.57</b>	<b>1.06-2.32</b>
HIV-positive, without treatment with ART	11	(5.1)	<b>3.63</b>	<b>1.39-9.45</b>

OR adjusted for age, marital status, age at first intercourse, the number of lifetime sexual partners, contraceptive methods used and smoking status, Papanicolaou test, CD4-count, HIV-viral-load and antiretroviral therapy or ART.

Values in bold =  $p < 0.05$ .

Pap test: Papanicolaou test, 95% CI: confidence interval, OR: odds ratio, HIV: Human Immunodeficiency Virus, ART: antiretroviral therapy, ASCUS: atypical squamous cells of undetermined significance, LSIL: low grade squamous intraepithelial lesion, HSIL: high grade squamous intraepithelial lesions.

\*Includes ASCUS, LSIL and HSIL.

\*\*Percentages were calculated regarding HIV-positive = 216 and HIV-negative  $n = 1,159$ .

Significant differences were observed concerning HPV type distribution regarding 200 to 349 cell/mm<sup>3</sup> CD4 count; HPV-18 had the greatest prevalence ( $p = 0.041$ ), followed by HPV-16 ( $p = 0.241$ ) and HPV-31 ( $p = 0.008$ ). Other differential distributions were observed for higher than 100,000 copies/mL HIV viral load; however, they were not statistically significant (Table 5).

## Discussion

HPV infection and multiple infection prevalence was found to be similar to that reported for Colombia in previous studies concerned with the heterogeneous female population [17].

HPV prevalence in the HIV-positive population was significantly higher than in the group of HIV-negative women. This agreed with other studies which found that HIV-related immunosuppression disabled an immune system response to concomitant infection [4,19].

High HPV infection prevalence in HIV-positive women has been reported in worldwide studies; there is 44% HPV infection in European countries such as Italy [5] and US studies have found 54%-73% prevalence [20,21], similar to that for Latin-American countries like Brazil where 48% to 68% HPV prevalence has been found in HIV-positive women [22,23].

Our results showed that multiple HPV infection occurred more frequently in HIV-positive women; this may have

been due to a deficient immune system [7], risky sexual behaviour engaged in by this particular group involving an increased exposure risk and higher reactivation rates regarding latent HPV infection [7,19]. Furthermore, it has been reported that the risk of acquiring later HPV infection becomes increased in HPV-infected women, which could contribute to high multiple infection frequency [24].

Some studies have shown the co-existence of more than one HPV type in the same organism [25]; however, the clinical relevance of being infected with multiple HPV types has not been clearly established. Multiple infection results have indicated that HPV facilitates persistence at the site of infection, leading to an increased risk of premalignant lesions progressing to CC [24,26].

Type-specific distribution revealed that HPV-16 was the most prevalent type in the population being studied; this was consistent with worldwide results to date [17,27,28]. High prevalence was found for the other HPV types being tested, such as HPV-31 in HIV-positive women and HPV-58 in the group of HIV-negative females; these viral types have been reported in Colombia in previous studies by our research group and others working in the field as being the most prevalent [17,29].

Such differential type distribution between groups (HIV-positive and HIV-negative) may have been partly due to immune system deficiency, thereby contributing towards the inefficient removal of the virus or evasion

**Table 4 Relative frequency and multivariate analysis of HPV infection in HIV- negative and HIV- positive women**

	HPV positive detection			
	n	(%)*	Adjusted OR	95% CI
<b>HIV status</b>				
Negative	513	(44.3)	Ref	
Positive	149	(69.0)	<b>2.37</b>	<b>1.69-3.34</b>
<b>HIV and one type only HPV (single infection)</b>				
HIV-negative, single infection	250	(21.6)	Ref	
HIV-positive, single infection	32	(14.8)	1.14	0.85-1.28
<b>HIV and &gt;1 type HPV (multiple infection)</b>				
HIV-negative, multiple infection	263	(22.7)	Ref	
HIV-positive, multiple infection	117	(54.2)	<b>3.43</b>	<b>2.39-4.92</b>
<b>HIV and HPV Clade 7</b>				
HIV-negative, HPV Clade 7	202	(17.4)	Ref	
HIV-positive, HPV Clade 7	77	(35.7)	<b>2.48</b>	<b>1.69-3.64</b>
<b>HIV and HPV Clade 9</b>				
HIV-negative, HPV Clade 9	454	(39.2)	Ref	
HIV-positive, HPV Clade 9	141	(65.3)	<b>2.47</b>	<b>1.73-3.53</b>
<b>HIV; immune status CD4 cell/mm<sup>3</sup> count</b>				
HIV-negative	513	(44.3)	Ref	
HIV-positive, < 200	63	(29.2)	<b>4.38</b>	<b>2.47-7.77</b>
HIV-positive, 200 - 349	39	(18.1)	<b>3.13</b>	<b>1.64-5.96</b>
HIV-positive, 350 - 500	29	(13.4)	<b>1.91</b>	<b>1.03-3.57</b>
HIV-positive, > 500	18	(8.3)	1.17	0.59-2.32
<b>HIV viral load copies/mL</b>				
HIV-negative	513	(44.3)	Ref	
HIV-positive, <4,000	121	(56.0)	2.41	0.67-3.48
HIV-positive, 4,000-99,999	15	(6.9)	2.43	0.96-6.14
HIV-positive, >100,000	13	(6.0)	<b>7.32</b>	<b>1.62-33.03</b>
<b>HIV and ART use</b>				
HIV-negative	513	(44.3)	Ref	
HIV-positive, treatment with ART	133	(61.6)	<b>2.46</b>	<b>1.72-3.51</b>
HIV-positive, without treatment with ART	16	(7.4)	<b>3.32</b>	<b>1.16-9.50</b>

OR adjusted for age, marital status, age at first intercourse, the number of lifetime sexual partners, contraceptive methods used and smoking status, Papanicolaou test, CD4-count, HIV-viral-load and antiretroviral therapy or ART. Abbreviations: 95% CI: confidence interval, OR: odds ratio, HIV: Human Immunodeficiency Virus, ART: antiretroviral therapy, HPV: Human Papillomavirus.

\*Percentages were calculated regarding HIV-positive = 216 and HIV-negative n = 1,159.  
 Clade 7: HPV-18 and -45; Clade 9: HPV-16, -31, -33 and -58. Values in bold = p < 0.05.

by certain types of HPV. The latter benefits the colonisation and persistence of some viral types (mainly oncogenic HPV) [7,8,30].

A positive association was seen in the current study for women having lower than 200 cell/mm<sup>3</sup> CD4 count with abnormal cytological findings, compared to those having higher CD4 count, thereby agreeing with a previous report [5]. Lower than 200 cell/mm<sup>3</sup> count indicates a deficient immune system and, together with HPV detection, could be used as predictors of pre-neoplastic cervical lesions [31].

Our groups' results showed that a lower than 500 cell/mm<sup>3</sup> CD4 count was associated with HPV detection, thereby reflecting the inability of HIV-positive women's immune systems to respond to opportunist infection. High HIV viral load and low CD4 cell counts could facilitate acquiring HPV infection [32,33].

The effect of antiretroviral drugs on the incidence of cervical lesions having a poor prognosis has not been clearly established; previous studies, such as the US WIHS (Women's Interagency HIV Study) cohort, have indicated that ART treatment for HIV positive women

**Table 5 Relative frequency of HPV infection and type-specific distribution in HIV-positive and HIV-negative women**

	HPV positive		HPV-type											
			HPV-16		HPV-18		HPV-31		HPV-33		HPV-45		HPV-58	
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
<b>HIV-positive (n = 216)</b>	149	69.0	100	46.3	66	30.6	71	32.9	40	18.6	19	8.8	44	20.4
<b>CD4 cell/mm<sup>3</sup> count</b>														
< 200	63	29.2	44	20.4	22	10.2	35	16.2	13	6.0	9	4.2	18	8.3
200 - 349	39	18.1	22	10.2	18	8.3	19	8.8	15	6.9	5	2.3	14	6.5
350 - 500	29	13.4	18	8.3	20	9.3	12	5.6	10	4.6	3	1.4	8	3.7
> 500	18	8.3	16	7.4	6	2.8	5	2.3	2	0.9	2	0.9	4	1.9
<b>HIV viral load copies/mL</b>														
<4,000	121	56.0	82	38.0	54	25.0	54	25.0	33	15.3	15	6.9	32	14.8
4,000-99,999	15	6.9	11	5.1	9	4.2	10	4.6	4	1.9	1	0.5	8	3.7
>100,000	13	6.0	7	3.2	3	1.4	7	3.2	3	1.4	3	1.4	4	1.9
<b>ART use</b>														
Yes	133	61.6	90	41.7	61	28.2	64	29.6	36	16.7	18	8.3	39	18.1
No	16	7.4	10	4.6	5	2.3	7	3.2	4	1.9	1	0.5	5	2.3
<b>HIV-negative (n = 1,159)</b>	513	44.3	222	19.2	118	10.2	151	13.0	104	9.0	98	8.5	177	15.3

HIV: Human Immunodeficiency Virus, ART: antiretroviral therapy, HPV: Human Papillomavirus.

has led to duration and progression becoming reduced [31]. Our group's results showed that women with and without treatment with antiretroviral drugs had a significant association with HPV detection and abnormal cytological findings; this could have been due to high HPV-16 prevalence, since it has been established that the effect of therapy involving ART on the appearance of cervical disease is reduced when infections by this viral type occur [31].

More lesions were detected in the HIV-positive group (mainly LSIL); studies have indicated that women having this type of immunosuppression have a higher incidence and prevalence of premalignant lesions caused by the immune system becoming unable to efficiently eliminate HPV infection. This also favours the virus' persistence in the body, ultimately contributing towards the development of this type of dysplasia [34,35].

Association studies have shown that HIV infection increases the risk of CC development (up to 22-fold) compared to such risk in the general population [19]. The natural history of CC development becomes altered in women having immunosuppression caused by HIV since the regression of lesions has been seen to decrease significantly compared to HIV-negative women [4,36].

This can be explained as immunosuppression leads to an alteration in local and systemic immune response, thereby preventing suitable clearance of HPV infection. This results in recurrent pre-neoplastic lesions and lower regression rates; however, it has not been clearly established whether this is the main mechanism [6,13,21].

Our results showed a significant association between infection involving clade 9 and HIV-positive women; this may have been partly due to HPV-16 in this clade being the most prevalent viral type in such population. This viral type has been shown to have an association with the risk of acquiring other HPV types; however, it has not been clearly established whether a higher risk of acquiring a phylogenetically-related type is associated with the risk of acquiring another type of HPV [7,24].

Interestingly, a positive trend was found between ASCUS cytology and HPV-DNA being detected. ASCUS cytology indicated that cytological-morphological changes were not benign; however, this did not meet the criteria for classification as squamous lesions, even though studies have shown that this type of cytological interpretation is highly related to characteristic subjective reading of Pap tests [37,38].

Studies have found that HPV detection in women having ASCUS has provided high sensitivity in detecting severe dysplasia and cancer [39]. This is why strengthening HPV molecular screening for detecting women having atypical ASCUS (first indicator of cervical abnormalities) may help in reducing the impact caused by CC on an immunocompetent population, as well as HIV-infected women who are at increased risk [40].

One of this study's limitations lay in the detection of 6 of the 15 HPV types described as being high risk. Even though the types identified in the present study are considered to be prevalent in the Colombian population, other types of high and low risk HPV should be included in future studies. This study's cross-sectional design did

not lead to determining a causal relationship between the immunodeficiency caused by HIV and/or the prevalence and persistence of HPV.

## Conclusions

There is limited information in Colombia regarding HPV infection in HIV-positive women. This is the first study in Colombia which has evaluated epidemiological profiles concerning HPV infection in both HIV-positive and HIV-negative women. The results further showed multiple HPV infection as an associated factor in HIV-positive women; however, further prospective studies are needed to determine the dynamics and follow-up patterns (i.e. 5-year follow-up) for these infections and their influence on the development of cervical dysplasia.

HR-HPV distribution in women should be studied to facilitate developing prevention and management strategies in the general population, prioritising HIV-positive women as they represent a special group due to their type of immunosuppression altering the natural course of CC development. The resulting information has contributed towards knowledge regarding HIV-HPV-related sexually-transmitted infections.

## Abbreviations

HPV: Human papillomavirus; HIV: Human immunodeficiency virus; ART: Antiretroviral therapy; HR-HPV: High risk human papillomavirus; LR-HPV: Low risk human papillomavirus; DNA: Deoxyribonucleic acid; CC: Cervical cancer; SIL: Squamous intraepithelial lesions; ASCUS: Atypical squamous cells of undetermined significance; LSIL: Low grade squamous intraepithelial lesion; HSIL: High grade squamous intraepithelial lesion; STD: Sexually-transmitted diseases; Pap test: Papanicolaou test; 95% CI: Confidence interval; OR: Odds ratio; SD: Standard deviation; PCR: Polymerase chain reaction.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

MC was involved in the design of the study, helped in the collection of clinical data, performed the molecular tests and analysis and drafted the manuscript. SCSL was involved in the design of the study, helped in the collection of clinical data, performed the molecular tests and analysis and drafted the manuscript. MM was involved in the design of the study, helped in the collection of clinical data, performed the molecular tests and analysis and drafted the manuscript. RS conducted statistical analyses and contributed to the manuscript. DPH was involved in the analysis and interpretation of data. ACP collected the clinical data and made a critical review of the manuscript. OS collected the clinical data and made a critical review of the manuscript. CP collected the clinical data and made a critical review of the manuscript. APP was involved in the analysis and interpretation of data. MEP co-designed the study and MAP co-designed the study, led the research project and proofread the final document. All authors read and approved the final manuscript.

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## Author details

<sup>1</sup>Fundación Instituto de Inmunología de Colombia, Cra. 50 # 26-20, Bogotá, Colombia. <sup>2</sup>School of Medicine and Health Sciences, Universidad del Rosario, Calle 14 # 6 - 25, Bogotá, Colombia. <sup>3</sup>Universidad Nacional de Colombia, Avenida Carrera 30 # 45, Bogotá, Colombia. <sup>4</sup>Northeastern University, 360 Huntington Ave, Boston, MA, USA. <sup>5</sup>Faculty of Natural and Mathematical Sciences, Universidad del Rosario, Calle 14 # 6 - 25, Bogotá, Colombia. <sup>6</sup>Asistencia Científica de Alta Complejidad S.A.S., Calle 45B # 24-25, Bogotá, Colombia. <sup>7</sup>Universidad Pública de Navarra, 31006 Pamplona, Spain.

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# Association of HIV status with infection by multiple HPV types

Milena Camargo<sup>1,2,\*</sup>, Luisa Del Río-Ospina<sup>1,\*</sup>, Sara Cecilia Soto-De León<sup>1,3,\*</sup>, Ricardo Sánchez<sup>1,4</sup>, Andrea Clemencia Pineda-Peña<sup>1</sup>, Otto Sussmann<sup>5</sup>, Manuel Elkin Patarroyo<sup>1,4</sup> and Manuel Alfonso Patarroyo<sup>1,6</sup>

1 *Molecular Biology and Immunology Department, Fundación Instituto de Immunología de Colombia, Bogotá DC, Colombia*

2 *PhD Program in Biomedical and Biological Sciences, School of Medicine and Health Sciences, Universidad del Rosario, Bogotá DC, Colombia*

3 *Universidad de Ciencias Aplicadas y Ambientales, Bogotá DC, Colombia*

4 *Medical School, Universidad Nacional de Colombia, Bogotá DC, Colombia*

5 *Asistencia Científica de Alta Complejidad S.A.S., Bogotá DC, Colombia*

6 *Basic Sciences Department, School of Medicine and Health Sciences, Universidad del Rosario, Bogotá DC, Colombia*

## Abstract

**OBJECTIVES** To identify the clinical and demographic characteristics of HIV-positive and HIV-negative women infected by multiple HPV types.

**METHODS** 1399 women participated in the study (240 HIV-positive and 1159 HIV-negative women). Samples were provided for Pap tests and for HPV detection and typing by PCR. Data were collected on HPV infection, frequency of multiple infection, and HPV type distribution. Odds ratios were reported from logistic regression models.

**RESULTS** Compared with HIV-negative women, HIV-positive women had higher frequencies of cervical abnormality (30% *vs.* 20.8%), higher HPV prevalence (68.3% *vs.* 51.3%) and were more commonly infected with multiple HPV types (78.7% *vs.* 44.3%). HPV-16 was the most common type detected in the study population, with other types showing variable associations with HIV status. Positive associations were observed between infection by multiple HPV types and HIV status, cervical abnormality and having had more than three pregnancies. The odds of multiple infection by HPV types were higher in HIV-positive women who used an intrauterine device, who had a history of abortions and who had HIV viral loads >100 000 copies/ml, whilst the odds were lower in women with >500 CD4 cells/mm<sup>3</sup>.

**CONCLUSIONS** HIV immunosuppression favours infection by multiple high-risk HPV types, mainly in women affected by low-grade squamous intraepithelial lesions. Antiretroviral therapy had no effect on infection by multiple HPV types. Risk factors related to progressive damage to the cervix were positively associated with infection by multiple HPV types in women living with HIV.

**keywords** human papillomavirus, human immunodeficiency virus, multiple infection, epidemiology, risk factor

## Introduction

High-risk (HR) human papillomavirus (HPV) types are highly prevalent sexually transmitted diseases (STDs) in women living with human immunodeficiency virus (HIV) [1]. HIV favours HPV persistence and modulates the expression of the HPV *E6* and *E7* genes, which are responsible for oncogenic transformation [2, 3]. HPV persistence is favoured as a result of HIV-induced impairment of host immune responses, which limits the host's ability to control infection and permits infection by

multiple HPV types. Infection by multiple HR-HPV types has been observed in a significant proportion of HIV-positive women [4].

Persistent infection by HR-HPV constitutes a risk factor directly associated with cervical cancer (CC) [5, 6]. HIV-associated immunosuppression is a key factor contributing to increased morbidity and mortality of this neoplasia [7]. The risk of developing lesions arising from HPV infection is significantly increased in HIV-positive women, with CC incidence approximately 20-fold higher than in HIV-negative women [8].

The clinical importance of infection by multiple HR-HPV types remains controversial. An intraepithelial lesion

\*These authors contributed equally to this work.

has traditionally been attributed to infection by a single HPV type [9]. Nevertheless, studies have revealed high frequency of multiple HPV infections in women with cervical lesions (low-grade squamous intraepithelial lesions (LSILs) or more serious lesions); and attributing an etiological relationship to a single genotype is difficult [9]. *In vitro* studies of the biology of multiple HPV infection have demonstrated a single cell's ability to be simultaneously infected by more than one HPV type; however, studies of multiple HPV infection in women with lesions have produced inconclusive results [9–11].

Studies of HIV-positive women have shown conflicting results regarding the prevalence of simultaneous infection by multiple HPV genotypes and their contribution to the risk of developing CC. Some studies have suggested that HPV types have different behaviours in immunosuppressed women (including HIV-positive women), thereby accelerating the progression of premalignant lesions to CC. By contrast, other studies have suggested that the biology of HPV infection is not influenced by HIV status [3, 12, 13]. Whilst HPV's natural history is clearly altered in HIV-positive women, more studies are needed to understand the risk factors for simultaneous infection by more than one HPV genotype and the populations at risk of multiple infection [8].

This study was thus aimed at understanding the determinants of infection by multiple HPV types in Colombian women with and without HIV infection. The information provided by this study should contribute towards improved understanding the factors involved in development of cervical neoplastic lesions and may help in developing effective interventions and prevention programs for CC. These are aimed at increasing the quality of life and life expectancy for the high-risk group of women living with HIV.

## Materials and methods

### Study population and ethical approval

This was a cross-sectional observational study; convenience sampling was used to enroll HIV-positive and HIV-negative women. Eligibility criteria included: (i) known HIV status reported by participating institutions with permission of each woman, (ii) clinically stable, (iii) not hospitalised at the time of their inclusion in this study, and (iv) agreed to participate in the study through signed informed consent. Women with alterations in the urogenital tract that affected the ability to obtain a sample were excluded.

All women included in this study were from Bogotá, Colombia's capital city. The HIV-positive women were recruited at the Asistencia Científica de Alta Complejidad

S.A.S. clinic, which specializes in treating this infection, whilst the HIV-negative women were attending three hospitals' sexual and reproductive health programs (Hospital de Bosa, Hospital de Fontibón and Hospital Engativá). Additional characteristics regarding the population and study design have been reported previously [14].

All protocols were approved by the ethics committee of each participating hospital and by the Fundación Instituto de Inmunología de Colombia's ethics committee. All women included in the study were informed of its purpose and procedures and voluntarily accepted an invitation to participate by signing an informed consent form. A structured questionnaire was used to compile sociodemographic information and data regarding participants' sexual behaviours.

This study included tests for the identification of cervical changes (Papanicolaou test) and detection of six HR-HPV types (molecular detection); as a cross-sectional study, both tests were conducted during a single visit. However, all results obtained (Pap test and molecular detection of HPV) were sent to each institution included in the study and interpreted by each institution's expert gynecologists, who defined the follow-up schemes following established recommendations according to the cervical cancer detection and control program of Colombia's health security system.

### Collecting, processing and detection of HPV DNA

The women were received by the head nurse responsible for each institution's promotion and prevention (P&P) program. Informed consent forms were signed and demographic data collected, then samples were obtained. Pap tests were conducted first according to Colombian technical standards and the Bethesda system was used for reporting cytological findings. The second sample provided was used for the molecular detection of HPV. A cervical mucosa scrape was obtained using a Cytobrush<sup>®</sup>, preserved and transported in 95% ethanol.

Total DNA was recovered by vigorous vortexing and extracted using a QuickExtract kit following the manufacturer's instructions. PCR was used for all molecular analyses. All samples were subjected to a first amplification specific for a segment of the human  $\beta$ -globin gene (GH20/PC04 primers) to verify DNA quality [15]. Samples showing positive amplification of  $\beta$ -globin were then subjected to generic HPV identification PCRs using three sets of primers (GP5+/6+, MY09/11 and pU1M/2R). Amplification was simultaneous and independent for all infected women, as using more than one set of generic detection primers ensures the robustness and sensitivity of epidemiological studies [15].

PCR type-specific identification assays were then carried out on all samples from HPV-positive women (positivity being defined as amplification by any of the generic primer sets). The methodological design included the detection of six genotypes (HPV-16, -18, -31, -33, -45 and -58) believed to be responsible for up to 85% of CC cases. These genotypes have been shown to have a wide distribution in Colombian populations [16, 17]. Independent PCR reactions were carried out with primers specific for each HPV genotype using previously described amplification conditions [15, 17].

### Statistical analysis

Quantitative variables were included in statistical analyses, along with their respective means and standard deviations (SD). Variables such as number of lifetime sexual partners and number of full-term pregnancies were treated as categorical variables and summarised using percentages and 95% confidence intervals (95% CI). Chi<sup>2</sup> or Fisher's exact tests were used to assess differences between proportions.

Associations between combinations of pairs of HPV types were analysed according to HIV status. Association strength was measured using odds ratios (ORs) adjusted for confounding variables (age, age at first sexual intercourse, pregnancies, history of other STDs, number of lifetime sexual partners, contraceptive methods used, history of abortions and smoking status).

Ordinal logistic regression models were used to estimating adjusted ORs, considering the number of infecting HPV types as the dependent variable (ranked 0–4, with 4 referring to infection by 4 or more genotypes). Clinical variables (CD4 cell count, HIV viral load and antiretroviral therapy (ART)) were added to the model describing HIV-positive women. HIV status, age, age at first sexual intercourse, total number of pregnancies, history of STDs, number of sexual partners, contraceptive method used, history of abortions and smoking status were considered independent variables. An additional model was constructed taking only HIV-positive women into account, to evaluate whether further independent variables (CD4 cell count in cells/mm<sup>3</sup>, HIV viral load in copies/ml and ART use) were associated with the dependent variable. STATA12<sup>®</sup> software was used for statistical analyses, with statistical significance assumed at an alpha-value of 0.05.

### Results

The study population consisted of 240 HIV-positive women and 1114 HIV-negative women who were eligible

and agreed to participate in the study between February 2007 and February 2013. The average age of women enrolled in the study was 37.5 years (SD: 10.6 years) and the average age at first sexual relationship was 17.7 years (SD: 3.4 years). The sociodemographic and clinical characteristics of study participants were stratified according to HIV status and are shown in Table 1.

Pap smears and molecular detection test results showed that 70 HIV-positive women (29.1%; 95% CI 23.4–35.3%) had abnormal cytological findings using the Bethesda system (Table 1) and 164 HIV-positive women (68.3%; 95% CI: 62.0–74.1%) tested positive for HPV. Of the 164 HPV positive-women, 129 (78.7%; 95% CI: 71.5–84.6%) were simultaneously infected by more than one HPV type (defined as multiple infection) (Table 1). Analysis of type-specific distribution revealed that HPV-16 had highest prevalence in the study population, followed by HPV-31 and -18; these prevalence figures showed statistically significant differences ( $P = 0.001$ ) (Figure 1a).

Compared with HIV-positive women, fewer HIV-negative women had cellular abnormalities and HPV infection. In total, 227 HIV-negative women (20.4%; 95% CI 18.0–22.8%) had some degree of cervical abnormality by Pap test, 486 were HPV positive (43.6%; 95% CI 39.0–44.9%) and 250 (51.4%; 95% CI 44.3–53.1%) were simultaneously infected by more than one HPV type (Table 1). In HIV-positive women, HPV-16 had the highest prevalence but the distribution of the remaining HPV genotypes differed from that in HIV-negative women. HPV-58 was the second most-prevalent type followed by HPV-31; these prevalence figures were statistically different from one another ( $P = 0.039$ ). The distributions revealed that HPV-45 had the lowest prevalence in the women studied here ( $P = 0.009$ ) (Figure 1b).

In HIV-positive women, HPV prevalence was higher in those having an abnormal Pap test result (76.4%) than in those without cervical lesions (64.9%) ( $P = 0.001$ ). A similar effect was observed in HIV-negative women, where HPV was detected more often in women with some sort of cervical abnormality (52.8% *vs.* 41.2%;  $P = 0.043$ ).

The number of genotypes involved in multiple infections was determined (simultaneous infection by 2, 3, 4 or more HPV genotypes) in women having some degree of cervical lesion (Figure 2). In HIV-positive women, infection by an increased number of HPV types was associated with an increase in lesion severity (mainly LSIL) ( $P = 0.046$ ) (Figure 2a); this contrasted with the HIV-negative group, where women with a cytological finding of atypical squamous cells of undetermined significance

Camargo *et al.* Multiple HPV types in HIV-positive women**Table 1** Demographic (A) and Clinic (B) characteristics of the HIV-positive ( $n = 240$ ) and HIV-negative women ( $n = 1114$ ) enrolled in the study

A. Demographic variable	HIV-positive ( $n = 240$ )	HIV-negative ( $n = 1114$ )
Age, years*	37.3 [20–73] SD = 10.6	37.5 [20–76] SD = 10.6
	$n$ (%)	$n$ (%)
Ethnicity		
Indigenous	3 (1.2)	2 (0.2)
Mestizo	232 (96.7)	1083 (97.2)
Afrocolombian	5 (2.1)	29 (2.6)
Age at first Intercourse, years		
<15	86 (35.9)	257 (23.1)
16–19	120 (50.0)	592 (53.1)
>19	34 (14.1)	265 (23.8)
Pregnancies		
None	17 (7.1)	57 (5.1)
1–3	174 (72.5)	751 (67.4)
>4	49 (20.4)	306 (27.5)
History of other STD		
No	152 (63.3)	629 (56.5)
Yes	88 (36.7)	485 (43.5)
Lifetime number of sexual partners		
1–2	100 (41.7)	753 (67.6)
>3	140 (58.3)	361 (32.4)
Contraceptive method		
None	100 (41.6)	636 (57.1)
Intrauterine device	20 (8.4)	196 (17.6)
Hormonal	12 (5.0)	176 (15.8)
Condom	108 (45.0)	106 (9.5)
Abortions		
No	134 (55.8)	703 (63.1)
Yes	106 (44.2)	411 (36.9)
Smoking status		
No	197 (83.0)	750 (67.3)
Yes	43 (17.9)	364 (32.7)
B. Clinical variable	HIV-positive ( $n = 240$ )	HIV-negative ( $n = 1114$ )
	$n$ (%)	$n$ (%)
Cytological findings		
Normal	170 (70.9)	887 (79.6)
ASCUS	26 (10.8)	121 (10.8)
LEIBG	40 (16.7)	93 (8.4)
LEIAG	4 (1.6)	13 (1.2)
HPV infection		
Negative	76 (31.7)	628 (56.4)
Positive	164 (68.3)	486 (43.6)
HPV infection status†		
Single Infection	35 (21.3)	236 (48.6)
Multiple Infection	129 (78.7)	250 (51.4)

**Table 1** (Continued)

B. Clinical variable	HIV-positive ( $n = 240$ )	HIV-negative ( $n = 1114$ )
	$n$ (%)	$n$ (%)
CD4 cell/mm <sup>3</sup> count		
<200	89 (37.1)	–
200–349	59 (24.6)	–
350–500	53 (22.1)	–
>500	39 (16.3)	–
HIV viral load copies/ml		
<1000	182 (75.8)	–
1000–4000	22 (9.2)	–
4000–99 999	22 (9.2)	–
>100 000	14 (5.8)	–
ART use		
With treatment	220 (91.7)	–
Without treatment	20 (8.3)	–

ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ART, antiretroviral therapy. SD, standard deviation; STD, sexually-transmitted diseases. \*Mean [Range].

†Denominator for this category was 164 HIV-positive and 486 HIV-negative women.

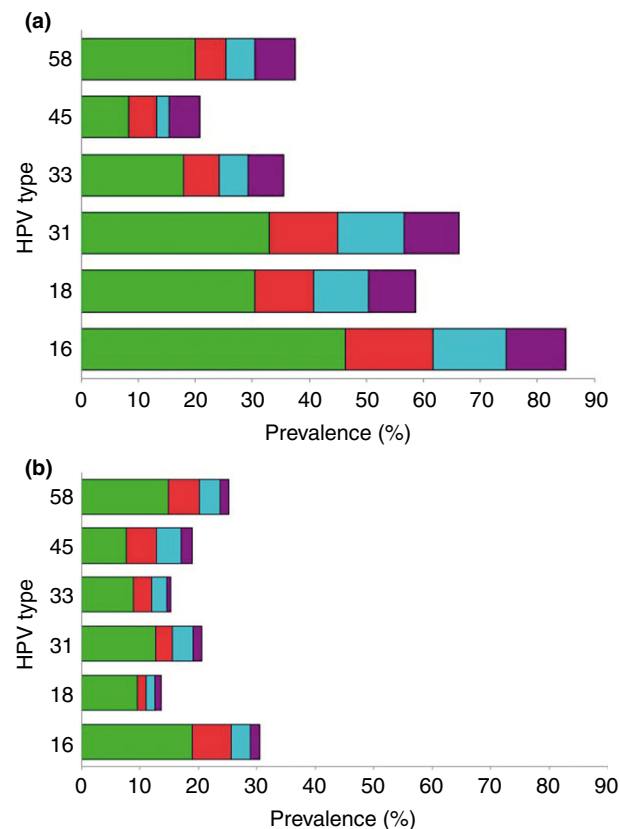
(ASCUS) had higher multiple infection frequency ( $P = 0.007$ ) (Figure 2b).

Considering that multiple infections occurred with high frequency in the study population, the frequency of simultaneous infection by 2, 3 and >3 HPV genotypes was evaluated separately (Figure 1). In HIV-positive women, HPV-16, -18 and -31 were the most prevalent types in all multiple infection scenarios (Figure 1a and Table S1).

In HIV-negative women, HPV-16 had the highest prevalence (6.7%) in simultaneous infections with only 2 genotypes, whilst HPV-45 had the highest prevalence (4.2%) in multiple infections by 3 or >3 types (2.0%) (Figure 1b and Table S1).

Crude and adjusted ORs were calculated to evaluate the association between pairs of HR-HPV types according to HIV status. Positive associations were observed for the HPV-16 and HPV-31 combination in HIV-positive women. This contrasted with HIV-negative women, where positive associations were found for all genotypes evaluated; these associations were strongest between HPV-16 and -18, HPV-45 and -18, and HPV-45 and -58 (Figure 3).

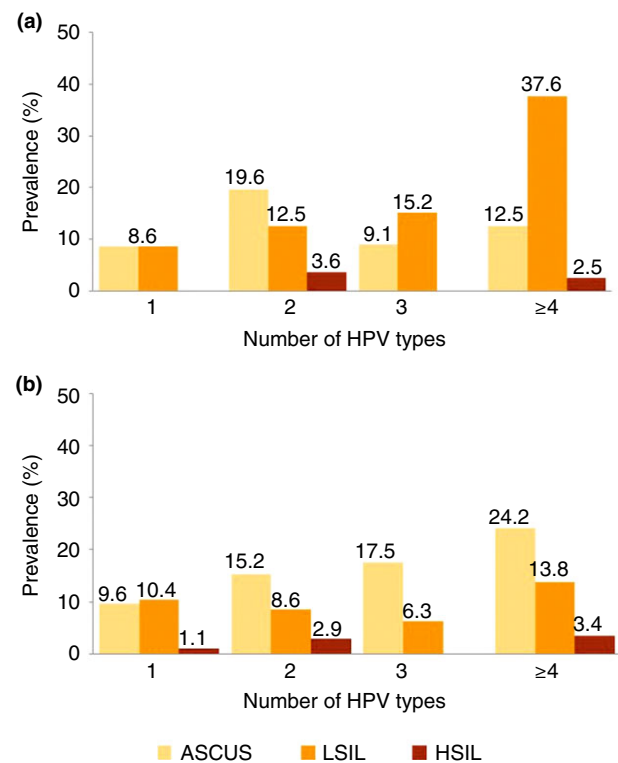
Ordinal regression analysis was used to establish whether an association was present between risk factors



**Figure 1** Relative frequency and distribution of multiple infection events for six high-risk HPV types according to HIV status (HIV-positive *vs.* HIV-negative patients). Green highlights each HPV type's relative frequency, red shows frequency of simultaneous detection of another genotype in the presence of each genotype, blue shows frequency of multiple infection with three genotypes for each genotype and purple shows the frequency of multiple infection with four or more genotypes for each genotype. [Colour figure can be viewed at [wileyonlinelibrary.com](#)].

and the number of HPV types in multiple infections. Several risk factors showed significant associations. Women aged 35–49 years old were less likely to be infected by multiple HPV types (adjusted OR: 0.67; 95% CI 0.52–0.87). By contrast, positive associations were observed for risk factors such as three previous pregnancies (adjusted OR 1.72; 95% CI 1.01–2.92) and HIV-positive status (adjusted OR 3.27; 95% CI 2.34–4.57) (Table 2).

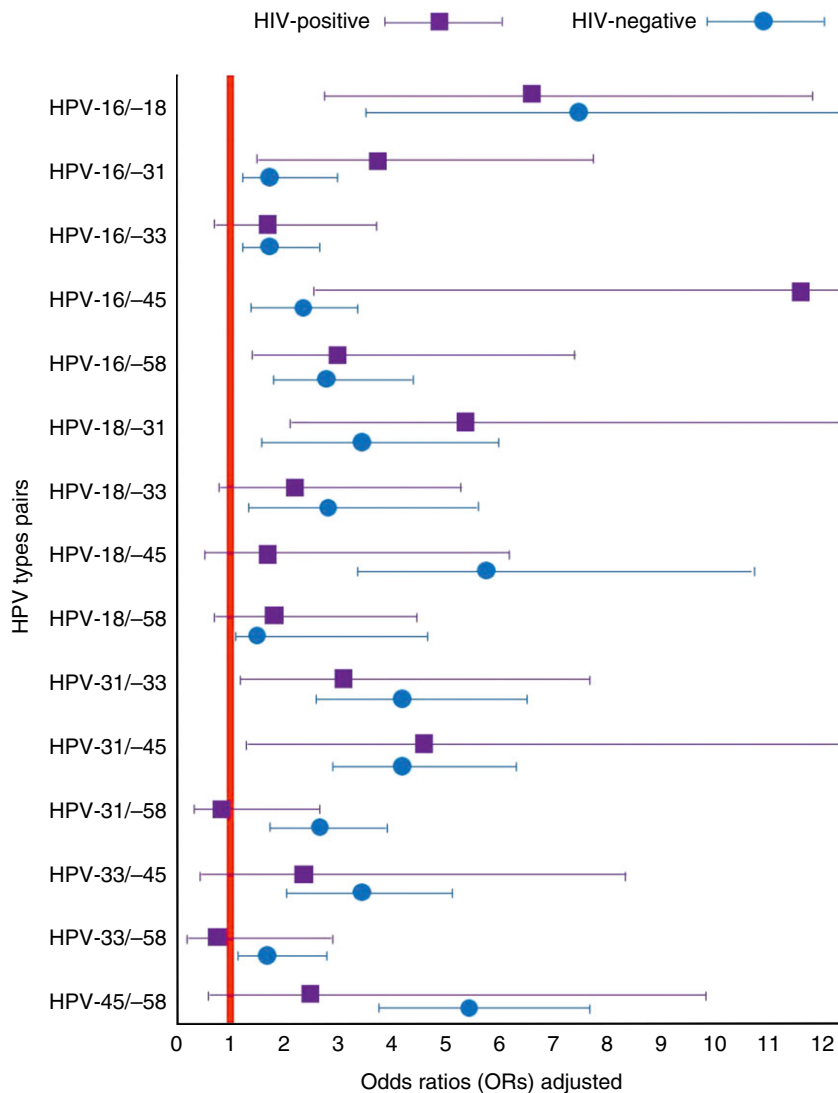
In a similar analysis involving only HIV-positive women other risk factors were identified associated with the number of HPV types in multiple infections. Positive associations were observed for women who had one to three pregnancies (adjusted OR 8.55; 95% CI 1.29–56.35) or more than four pregnancies (adjusted OR 3.65;



**Figure 2** Distribution of the number of HPV genotypes in HIV-positive and HIV-negative women according to cytological findings. [Colour figure can be viewed at [wileyonlinelibrary.com](#)].

95% CI 1.38–9.66), used an intrauterine device (adjusted OR 5.98; 95% CI 1.15–36.19) or had a history of abortions (adjusted OR 2.63; 95% CI 1.17–5.89). The clinical characteristics (determined exclusively in HIV-positive women) showed that a viral load >100 000 copies/ml (adjusted OR 7.76; 95% CI 1.64–63.19) was strongly associated with multiple infection; by contrast, women with CD4 cell counts >500 cells/mm<sup>3</sup> were at lower risk of multiple infection (adjusted OR 0.38; 95% CI 0.13–0.98) (Table 3).

The association between cytological findings and the number of HPV types was evaluated in the study population. The results highlighted that women with ASCUS (adjusted OR 1.96; 95% CI 1.41–2.73), LSIL cytological findings (adjusted OR 1.71; 95% CI 1.20–2.44) or high-grade squamous intraepithelial lesion (HSIL) cytological findings (adjusted OR 2.43; 95% CI 1.03–5.72) had a higher probability of being infected by multiple HPV types (Table 4A). This positive association was maintained in women with LSIL cytological findings (adjusted OR 2.97; 95% CI 1.29–6.83) in the group of women living with HIV (Table 4B).



**Figure 3** Number of infection pairs and odds ratios (ORs) according to pairwise combinations of specific HPV types and HIV status. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)].

## Discussion

This study was intended to help understand the factors and determinants involved in simultaneous infection by more than one HPV genotype in women living with HIV, as these viruses share the same transmission route. We found that HPV infection and infection by multiple HPV types was more common in HIV-positive women (Table 1 and Figure 1a). This was expected since HIV-associated immunosuppression leads to low pathogen clearance due to reduced efficiency of cell-mediated, systemic and local immunity, thereby influencing the persistence of more than one viral genotype in the cervical epithelium [12, 18].

The high frequency of infection by multiple HPV types reported here agrees with previous work in other regions

with high HIV prevalence [8], bearing in mind that both infections are sexually transmitted. HPV infection is favoured by HIV-immune responses generated during seroconversion as well as HIV-associated immunosuppression, which contributes to reactivation of latent stage HPV infections and to persistence of multiple cervical HPV types [4, 6].

HPV-16 had the highest prevalence in the study population (regardless of HIV status) (Figure 1); nevertheless, the results regarding this HPV type's contribution to CC development in HIV-positive women have been contradictory. The Women's Interagency HIV Study (WIHS) found that this HPV type had lower prevalence and weak association with development of high-grade cervical abnormalities in HIV-positive women (compared with

Camargo *et al.* Multiple HPV types in HIV-positive women**Table 2** Risk factors regarding the women included in this study ( $n = 1354$ ) associated with the amount of HPV types

	OR*	95% CI
HIV status		
Negative	Reference	
Positive	<b>3.27</b>	<b>2.34–4.57</b>
Age		
20–34	Reference	
35–49	<b>0.67</b>	<b>0.52–0.87</b>
>49	1.33	0.92–1.93
Age at first intercourse		
<15	Reference	
16–19	0.88	0.68–1.14
>19	1.06	0.75–1.43
Pregnancies		
None	Reference	
1–3	<b>1.72</b>	<b>1.01–2.92</b>
More of 4	1.29	0.97–1.71
History of other STD		
No	Reference	
Yes	1.14	0.87–1.41
Lifetime number of sexual partners		
1–2	Reference	
More of 3	1.19	0.94–1.50
Contraceptive method		
None	Reference	
Intrauterine device	1.03	0.69–1.55
Hormonal	0.82	0.54–1.24
Condom	1.07	0.77–1.49
Abortions		
No	Reference	
Yes	1.21	0.95–1.54
Smoking status		
No	Reference	
Yes	0.87	0.69–1.10

Values in bold =  $P < 0.05$ . CI, confidence interval; STD, sexually-transmitted diseases; ART, antiretroviral therapy.

\*OR adjusted for HIV status, age, age at first intercourse, pregnancies, history of other sexually-transmitted diseases, the number of lifetime sexual partners, contraceptive methods used, history of abortions and smoking status.

immunocompetent ones) [19]. By contrast, studies in countries with heterogeneous populations have indicated that HPV-16 continues to have the highest prevalence (regardless of HIV status) and contributes towards development of cervical lesions in the population living with HIV [2, 20, 21].

The results of several studies have shown that non-HPV-16 types have high frequencies in the HIV-positive population and could play an important role in high-grade cervical lesion development. Bearing in mind that non-HPV-16 genotypes are less efficient at evading the immune system, the impact of HIV-related immunosuppression favours the persistence of these less common

**Table 3** Multivariable ordinal logistic regression with all factors included in the model in HIV-positive women ( $n = 240$ )

	OR*	95% CI
Age		
20–33	Reference	
34–49	0.91	0.41–2.04
>49	2.24	0.63–7.90
Age at first intercourse		
<15	Reference	
16–19	0.86	0.40–1.83
>19	0.67	0.20–2.17
Pregnancies		
None	Reference	
1–3	<b>8.55</b>	<b>1.29–56.35</b>
More of 4	<b>3.65</b>	<b>1.38–9.66</b>
History of other STD		
No	Reference	
Yes	1.04	0.49–2.20
Lifetime number of sexual partners		
1–2	Reference	
More of 3	1.67	0.74–3.61
Contraceptive method		
None	Reference	
Intrauterine device	<b>5.98</b>	<b>1.15–36.19</b>
Hormonal	0.72	0.14–3.61
Condom	1.78	0.81–3.91
Abortions		
No	Reference	
Yes	<b>2.63</b>	<b>1.17–5.89</b>
Smoking status		
No	Reference	
Yes	1.68	0.68–4.14
CD4 cell/mm <sup>3</sup> count		
<200	Reference	
200–349	1.13	0.44–2.89
350–500	0.79	0.32–1.98
>500	<b>0.38</b>	<b>0.13–0.98</b>
HIV viral load copies/ml		
<1000	Reference	
1000–4000	1.38	0.39–4.89
4000–99 999	5.83	0.25–9.33
>100 000	<b>7.76</b>	<b>1.64–63.19</b>
ART use		
With treatment	Reference	
Without treatment	1.88	0.47–7.54

Values in bold =  $P < 0.05$ . CI, confidence interval; STD, sexually-transmitted diseases; ART, antiretroviral therapy.

\*OR adjusted for age, age at first intercourse, pregnancies, history of other sexually-transmitted diseases, the number of lifetime sexual partners, contraceptive methods used, history of abortions and smoking status, CD4-count, HIV-viral-load and antiretroviral therapy (ART).

HPV types in the host and reduces clearance rates [3, 11, 22]. This suggests the need to include new genotypes in vaccination schemes, which would represent an

**Table 4** Multivariable ordinal logistic regression of cytological findings associated with the amount of HPV types

	A. All Women ( <i>n</i> = 1354)		B. HIV-positive ( <i>n</i> = 240)	
	OR*	95% CI	OR*	95% CI
Cytological findings				
Normal	Reference		Reference	
ASCUS	1.96	1.41–2.73	1.61	0.69–4.16
LSIL	1.71	1.20–2.44	2.97	1.29–6.83
HSIL	2.43	1.03–5.72	1.54	0.23–5.95

CI, confidence interval; ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.

\*OR adjusted for HIV status, age, age at first intercourse, pregnancies, history of other sexually-transmitted diseases, the number of lifetime sexual partners, contraceptive methods used, history of abortions and smoking status.

appropriate strategy for reducing mortality caused by this neoplasm in HIV-positive women.

The present study demonstrated that the risk of acquiring multiple HPV infections was lower in women aged 35–49 years (Table 2), in agreement with previous descriptions of the natural history of infection by this virus [23, 24]. Number of births/parity was found to be positively associated with HPV multiple infection. The magnitude of this association was higher in HIV-positive women, which could be explained by the hormonal changes occurring with each pregnancy, favoring efficient viral replication [25].

The HPV life cycle in the host begins with its success in reaching the stratified squamous epithelium's basal cells, requiring a mechanical rupture to facilitate access [26, 27]. The results presented here showed positive associations between infection by multiple HPV types and use of an intrauterine device as well as a history of abortion in HIV-positive women (Table 3). Both of these factors result in epithelial inflammation, oxidative stress and cell damage, thereby facilitating mechanical rupture [28, 29] and providing HPV access to basal cells. The results suggested that exposure to these risk factors favoured the acquisition of multiple HPV types in HIV-positive women.

HPV penetration of the epithelium is favored by the expression of HIV proteins, leading to alteration in the cervix's normal architecture [18, 27]. It has been shown *in vitro* that expression of the HIV proteins Tat and gp120 increases transcription of genes encoding the HPV E6 and E7 proteins [24, 30]. A positive association between HIV viral loads greater than >100 000 copies/ml and infection by multiple HPV types was found here

(Table 3), suggesting that HIV replication facilitates the coexistence and replication of multiples HPV types in cervical tissues.

The results also revealed a protective effect against multiple infection events in women having more than 500 CD4 cells/mm<sup>3</sup>, indicating that the immune response is the key factor regarding HPV infection clearance. Immunity mediated by these cells favours efficient HPV clearance, limiting the pathogenesis of these infections [2, 31].

ART increases life expectancy in HIV-positive women since its consistent use leads to higher CD4 counts and lower viral loads; however, its usefulness in preventing CC development is still controversial. ART seems to favor elimination of HPV infection and also reduces the chance of acquiring new infections; however, ART does not seem to be able to completely restore immunity, as shown by the high HPV infection frequency in HIV-positive women. This suggests that ART has minimal effect on the natural history of CC infection and its development [2]. Our results indicated that using ART did not appear to have any additional effect on CD4 cell count and/or viral load; and no association between ART and HPV infection dynamics was observed in the HIV positive women in this study.

Strong associations between the HR-HPV pairs evaluated were observed in HIV-negative women, regardless of their phylogenetic relationships (Figure 3) [15]. There were fewer such associations between combinations of HPV genotypes observed in HIV-positive women; however, some were conserved including the association between HPV-16 and -31, suggesting that simultaneous infection by more than one HR-HPV genotype might occur differently in an immunosuppressed population [3]. Previous reports of HIV-positive women have indicated that whilst this infection facilitated HPV acquisition, specific HPV types might favor infection by additional HPV types, thereby promoting viral integration and cervical carcinogenesis [3].

The association between HPV multiple infection and low-grade cervical lesions occurred regardless of HIV status (Figure 2). Regarding HR-HPV multiple infection dynamics, two stages of the viral cycle (episomal replication and integration) were found to simultaneously promote the expression of proteins whose interactions might inhibit oncogenesis. Such negative regulation has been described for the E2-HPV-16 protein which blocks E6/E7-HPV-16/18 transcription, reducing integration and the appearance of high-grade cervical lesions [32, 33].

Nevertheless, a lack of follow-up of individuals with multiple HPV infections has limited a detailed relationship being established between multiple infections, HIV

status and the development of cervical lesions with poor prognosis. Prospective studies are needed to address these issues in detail. These would enable characterisation of the chronology of infection events and the natural history of cervical disease in HIV-positive women.

### Conclusion

The results of this study should contribute towards understanding the natural history of CC development in HIV-positive women. The results indicated that HIV influences multiple HPV infection behaviour and dynamics. Further knowledge is required regarding the factors involved in the complex interactions between these two viruses and how their relationship influences the progression of pre-neoplastic lesions. Factors such as using ART appear to have no significant effect on the acquisition of multiple HPV types. Understanding the link between these viruses will provide better information for CC P&P programs regarding this population group and aid evaluation of the impact and effectiveness of prophylactic HPV vaccines currently being marketed.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Percentage of multiple infection events for six high-risk HPV types according to HIV status.

**Corresponding Author** Manuel Alfonso Patarroyo, Basic Sciences Department, School of Medicine and Health Sciences, Universidad del Rosario, Bogotá DC, Colombia. E-mail: mapatarr.fidic@gmail.com

## CONCLUSIONES, PERSPECTIVAS Y RECOMENDACIONES

### Conclusiones

En este proyecto, se realizó la cuantificación de la carga viral de los tipos oncogénicos más frecuentes en nuestro país (VPH-16, -18, -31, -33, -45 y -58), por lo que se pudo evaluar la influencia del número de copias de VPH en las lesiones de mal pronóstico y la coexistencia con *C. trachomatis*. Los datos reportados aquí sugieren que la carga viral se asocia con la presencia de lesiones a nivel cervical, por lo que medir el número de copias de VPH, puede mejorar el valor predictivo en la identificación de mujeres en riesgo de desarrollar lesiones precursoras de CC; sin embargo, el alcance de la cuantificación depende del tipo viral detectado. Estos hallazgos alientan a la implementación de la medición de la carga viral, lo que podría complementar los métodos usados rutinariamente en los programas de tamización cervical.

En cuanto a la coexistencia entre ITS, se puede concluir que estos eventos ocurren con alta frecuencia en población sexualmente activa, debido posiblemente a que comparten la misma ruta de transmisión, beneficiándose así mutuamente de esta interacción; otra explicación puede ser el hecho de compartir el tropismo por las mismas células diana, a pesar de exhibir patrones de infección particulares para cada microorganismo. En cuanto a la dinámica de las infecciones por *C. trachomatis*, se observó que en mujeres que iniciaron el estudio sin esta infección bacteriana, al cabo de un año de seguimiento, la mitad de la población ya había adquirido la bacteria. Por otra parte, las mujeres que iniciaron infectadas requirieron de un tiempo aproximado de seis meses para depurar la infección.

Se observó que el número de copias de VPH constituye un factor relacionado con el curso clínico de las infecciones por *C. trachomatis*, puesto que variaciones en la carga viral tenían un efecto en la resolución o persistencia de dichas infecciones, dinámica que fue dependiente del tipo viral presente. Adicionalmente, otros factores como el número de compañeros sexuales, antecedentes de abortos, historia de infecciones de transmisión sexual y edad mayor a 45 años, se asociaron con el desenlace de infecciones bacterianas. En los

últimos años, se ha descrito que la coexistencia entre VPH y *C. trachomatis* contribuye a un efecto sinérgico, el cual favorece la persistencia de estas infecciones en el hospedero, trayendo como consecuencia daño continuo y progresivo de la arquitectura del cérvix.

Por otra parte, se observó que mujeres que conviven con VIH, presentan un mayor riesgo de detección de VPH, detección simultánea de múltiples tipos de VPH y cambios morfológicos-cervicales. Adicionalmente, los resultados mostraron que el número de copias de VIH y el recuento de células CD4, podrían favorecer un mayor aparición de infecciones por VPH y la presencia simultánea de múltiples tipos. Por otro lado, el uso de la terapia antirretroviral (ART), mostró tener un efecto solo para la infección por VPH. Se requiere un mayor conocimiento sobre los factores involucrados en la interacción compleja entre estos virus, así como la influencia de esta relación en la progresión de las lesiones preneoplásicas. Esta información es relevante para la priorización de estrategias de tamización en esta población.

La asociación entre VPH y CC se encuentra claramente establecida; sin embargo, sólo una fracción de las mujeres que cursa con infección viral desarrolla lesiones cervicales, por lo que establecer la influencia de elementos adicionales en este proceso, es clave en la comprensión de la dinámica de la enfermedad. Este estudio mostró que factores comportamentales influyen en la adquisición de las ITS, por lo que acompañar los programas de tamización cervical con estrategias educación podría contribuir en la sensibilización (en términos de prevención) de la población femenina. Adicionalmente, nuestros resultados sugieren que la coexistencia con otros patógenos, contribuye significativamente con el curso clínico de las infecciones por VPH, por lo que estos hallazgos plantean nuevos retos en el abordaje e implementación de estrategias que incluyan la vigilancia y control de patógenos que comparten la ruta de transmisión.

### **Perspectivas y Recomendaciones**

Con la realización de este estudio, surgió interés por otros aspectos relacionados con el CC y la infección por VPH, que merecen ser objeto de nuevas investigaciones. Teniendo en cuenta que los resultados mostraron asociaciones entre la carga viral y anormalidad

cervical, sería relevante para futuros estudios aumentar el número de mujeres con lesiones de mayor gravedad (LEIBG, LEIAG), puesto que en este estudio las mujeres con hallazgos anormales se reunieron en un único grupo de anormalidad, lo que pudo llevar a generalizar los resultados obtenidos. Por lo tanto, un análisis de la dinámica de la carga viral del VPH de acuerdo con cada hallazgo colposcópico, podría ser más informativa.

El presente estudio contempló algunos factores de riesgo sociodemográficos y del comportamiento sexual; no obstante, para futuros estudios, valdría la pena evaluar factores intrínsecos del hospedero, como los relacionados con la respuesta inmune, ya que se ha descrito que polimorfismos en los genes del HLA clase I (HLA-A, -B y -C) y clase II (HLA-DR y -DQ), se asocian con el desarrollo de lesiones neoplásicas e infección por VPH. Esta información permitiría aclarar el vínculo entre una respuesta inmune ineficiente y la susceptibilidad a la infección por VPH.

En cuanto a *C. trachomatis*, información con la que no se contó en el presente estudio debe ser tomada en cuenta a futuro, como la referente a si las mujeres (o sus parejas) cuentan con tratamiento antibiótico, la presentación de síntomas relacionados con la presencia bacteriana, la cuantificación de la carga bacteriana y la genovariante en infección. Con respecto a este último factor de riesgo y teniendo en cuenta el trabajo adicional realizado en el capítulo 1, se debería avanzar hacia la unificación de los marcadores; es así como nuestro estudio permite proponer tres genes como claves a incluir en un esquema de tipificación alternativo, cuyo uso debe ser inicialmente validado en muestras clínicas.

Finalmente, para aumentar la información referente a la dinámica de la infección por VPH en población que convive con VIH, valdría la pena plantear estudios prospectivos que permitan caracterizar la cronología de los eventos de infección y coinfección, así como el desarrollo de lesiones de mal pronóstico relacionadas con VPH. Adicionalmente, teniendo en cuenta que el estudio de cohorte encontró que el número de copias de VPH tiene un efecto en la coexistencia con otras ITS, sería relevante llevar a cabo su cuantificación en mujeres VIH positivas.

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