VIRUS DE LA LEUCOSIS BOVINA (VLB) Y EVIDENCIAS DE SU POTENCIAL ZOONÓTICO

NURY NATHALIA OLAYA GALÁN B.Sc, M.Sc

TESIS DOCTORAL

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MARÍA FERNANDA GUTIÉRREZ, M.Sc, Ph.D
Pontificia Universidad Javeriana
Director

MANUEL ALFONSO PATARROYO, M.D., Ph.D
Fundación Instituto de Inmunología de Colombia (FIDIC)
Co-director

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MARÍA FERNANDA GUTIÉRREZ, M.Sc, Ph.D
Directora tesis doctoral

MANUEL ALFONSO PATARROYO, M.D., Ph.D
Co-director tesis doctoral

CAROLINA CERIANI, M.Sc, Ph.D
UNICEN – Argentina
Jurado

MARIA CRISTINA NAVAS, M.Sc, Ph.D
Universidad de Antioquia - Medellín
Jurado

LUISA MATHEUS, M.Sc, Ph.D
Universidad del Rosario - Bogotá
Jurado

UNIVERSIDAD DEL ROSARIO
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2021

DEDICATORIA

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LISTA DE ABREVIATURAS

AP3D1 - Adaptor Related Protein Complex 3 Subunit Delta 1

BLAST - Basic Local Alignment Search Tool

BRCA1/2 - breast cancer 1/2 gene

CAT1/SLC7A1 - Cationic amino acid transporter 1/Solute Carrier Family 7 Member 1

FIDIC - Fundación Instituto de Inmunología de Colombia (FIDIC)

FLK - Fetal Lamb Kidney

gp51 - Glicoproteína 51

HBV - Hepatitis B Virus

HCV - Hepatitis C Virus

HPV - Human Papiloma Virus

HTLV - Human T Lymphotrophic Virus

ICA - Instituto Colombiano Agropecuario

KSHV - Kaposi Sarcoma Herpes Virus

LEB - Leucosis Enzoótica Bovina

MMTV - Mouse mammary tumor virus

OIE - Organización Mundial de Sanidad Animal

OR - Odd ratio

PALB2 - partner and localizer of BRCA2

PCR - Polymerase Chain Reaction

PUJ - Pontificia Universidad Javeriana

SARS-CoV-2 - Severe acute respiratory syndrome coronavirus 2

VEB - Virus de Epstein-Barr

VLB - Virus de la Leucosis Bovina

RESUMEN

El Virus de la Leucosis Bovina (VLB) es un retrovirus con capacidad oncogénica que se caracteriza por ocasionar infecciones persistentes en el ganado vacuno, lo que conlleva a procesos crónicos de linfocitosis y en los casos más avanzados puede generar leucemias o linfomas en los animales. En las últimas décadas, este virus ha despertado la inquietud en el gremio científico debido a diferentes estudios que reportan la detección del provirus en muestras de origen humano, y se propone como un potencial factor de riesgo para el desarrollo del cáncer de mama. Hasta el momento se desconoce cuál es la vía de transmisión del virus al humano, pero se plantea que podría tratarse de una infección zoonótica debido al aumento de las interfaces de contacto entre los animales y los humanos. Asimismo, el virus se ha encontrado en múltiples especies animales, lo que lo propone como un agente versátil con un potencial alto de diseminación en su entorno natural ya que debido a la dificultad en el diagnóstico de rutina en campo y a la falta de vacunas, se ha reportado un aumento en las tasas de prevalencia del virus, el cual pasa desapercibido en las poblaciones animales aumentando las tasas de propagación y diseminación. En esta tesis doctoral se planteó un estudio enfocado en determinar la presencia del virus tanto en mujeres colombianas como en diferentes especies animales para aportar evidencias del potencial zoonótico del virus y actualizar el conocimiento de este en Colombia. Se realizó un estudio de casos y controles para identificar si el virus se encontraba asociado al cáncer de mama en una población de mujeres colombianas, y se realizaron avances en la caracterización del potencial zoonótico del virus al evaluar marcadores compartidos entre el virus circulante en diferentes especies animales y en los humanos, así como aproximaciones experimentales de la capacidad de infección del virus en células humanas y la identificación de su potencial receptor celular. Si bien aún es necesario realizar más estudios para entender en su totalidad el impacto que tiene el VLB en el humano, con los resultados obtenidos en esta investigación se realizaron aportes al conocimiento del virus en el país al tener las prevalencias actualizadas del VLB en el ganado bovino colombiano, se identificó el virus como un potencial factor de riesgo para el cáncer de mama y se generó un avance en el conocimiento con respecto al potencial zoonótico del virus abordado desde los principios de las infecciones zoonóticas, como la identificación de hospederos accidentales, identificación de marcadores compartidos entre especies, identificación de potenciales vías de diseminación y evidencias experimentales de la infección del virus en el humano.

ABSTRACT

Bovine Leukemia Virus (BLV) is a retrovirus with oncogenic potential which causes persistent infections in cattle, leading to chronic lymphocytosis processes, which could evolve into leukemias and/or lymphomas in advanced stages of the disease in cattle. In recent decades, this virus has aroused interest in the scientific community due to different studies reporting evidence of the provirus in humans, and its proposal as a potential risk factor for breast cancer development. However, its transmission pathway to humans remains uncertain, but it is suggested that it could be a zoonotic infection due to the increase of potential contact interfaces between animals and humans. Likewise, the virus has been found in multiple animal species, which proposes it as a versatile agent with a high potential for dissemination in its natural environment. Due to the difficulty in the diagnosis in the veterinary field, as well as the lack of vaccines, an increase in the prevalence rates of the virus has been reported worldwide, what makes the virus to remain unnoticed in animal populations, increasing the rates of propagation and dissemination. In this PhD research, it was proposed a study focused on the detection of the virus in Colombian women as well as in different animal species to provide evidence of the zoonotic potential of the virus and to contribute to the current knowledge of BLV in Colombia. A case-control study was carried out to identify whether the virus was associated with breast cancer in a population of Colombian women, and progress was made in the characterization of the zoonotic potential of the virus by evaluating shared biological markers between animal species and humans, and by experimental approaches to the virus's infection capacity in human cells and its potential cellular receptor. Although it is still necessary to carry out more studies to fully understand the impact of BLV in humans, the results obtained in this research contributed to the knowledge of the virus in the country by providing updates in the current status of BLV in Colombia, in terms of its prevalence rates in the Colombian cattle, identification of the virus as a potential risk factor for breast cancer development in Colombian women, and giving advances in the knowledge of its zoonotic potential approached from the principles of zoonotic infections, including the identification of accidental hosts, identification of shared biological markers between species, identification of potential dissemination routes and experimental evidence of the infection in humans.

VIRUS DE LA LEUCOSIS BOVINA (VLB) Y EVIDENCIAS DE SU POTENCIAL ZOONÓTICO

1. INTRODUCCIÓN

El virus de la leucosis bovina (VLB) es un retrovirus exógeno con capacidad oncogénica perteneciente al género deltaretrovirus el cual infecta naturalmente al ganado bovino. El VLB es el agente causal de leucosis enzóotica bovina (LEB), que se describe como una infección crónica en el ganado vacuno relacionada con una linfocitosis persistente y con el eventual desarrollo de leucemias y/o linfomas en los estadios más avanzados de la enfermedad. Sin embargo, tan solo el 5 y el 10% de los animales infectados desarrollan los estadios más avanzados, lo que hace que los animales persistentemente infectados con VLB permanezcan en los hatos ganaderos sin sintomatología clínica aparente o muy leve, lo que dificulta los procesos de control y favorece la diseminación del virus a los animales no infectados (1). La infección del VLB conlleva a complicaciones inmunológicas en los animales infectados aumentando la susceptibilidad a infecciones secundarias en el ganado como la tuberculosis bovina (2) o la mastitis (3,4). Adicional a esto, se ha reportado disminución en los rendimientos de los animales en cuanto a la producción de leche, problemas de reproducción, pérdida de peso, y otros hallazgos clínicos con implicaciones en la salud de los animales y su sistema inmune generando grandes pérdidas económicas para los ganaderos (5–8).

El VLB está distribuido a nivel mundial con prevalencias que oscilan entre un 10 y un 90% según las regiones geográficas analizadas (9). La presencia del virus tiene un impacto especial en las Américas ya que no existen programas de control oficial ni de erradicación a diferencia de Europa, y esto hace que se mantengan prevalencias por encima del 70-80% en la mayoría de los países de esta región. En Europa, los programas de erradicación y control han permitido eliminar al virus de más de 22 países, y en la actualidad la comercialización del ganado es altamente controlada para evitar que reingrese el virus a la región (10–13). Además de los bovinos, el VLB se ha encontrado de manera natural en otras especies incluyendo búfalos, ovejas, cabras y alpacas (14–18), lo que dificulta implementar y ejecutar estrategias de prevención y control en la industria pecuaria aumentando las tasas de prevalencia del virus, sobre todo en países en donde no se hace un

diagnóstico de rutina a los animales para identificar la presencia del VLB y hacer seguimiento de la infección en campo.

La presencia del virus en múltiples especies lo ha propuesto como un agente versátil, debido a las evidencias encontradas tanto en su entorno natural como en condiciones experimentales (15,19,20), en las cuales se ha logrado infectar a nivel de laboratorio animales como cabras, conejos, pollos y cerdos bajo condiciones controladas (21), así como líneas celulares provenientes de múltiples especies incluyendo el humano y células diferentes al linfocito B, las cuales se describen como las células blanco en el bovino (22–28). Estas evidencias apoyan la hipótesis de la capacidad del virus de infectar una amplia gama de hospederos y se propone que esto puede ser debido a receptores compartidos que permiten el ingreso del virus a células de otras especies. Hasta el momento se plantean como potenciales receptores celulares dos proteínas involucradas en el transporte celular, la AP3D1 y CAT1/SLC7A1 (29,30), de sus siglas en inglés *'Adaptor Related Protein Complex 3 Subunit Delta 1'* y *'Cationic amino acid transporter 1/Solute Carrier Family 7 Member 1'* respectivamente, las cuales son comunes en los mamíferos y comparten altos porcentajes de identidad entre especies.

Aparte de las evidencias del VLB en otras especies diferentes al bovino, en las últimas décadas se ha registrado la presencia del virus en el humano a través de la identificación de marcadores biológicos del virus como la presencia de fragmentos génicos en estadio proviral (31–33), proteínas (34) e incluso se ha reportado la presencia de anticuerpos reactivos contra el virus como muestra de exposición al VLB (35). Durante varios años se ha tratado de mostrar las implicaciones del VLB en el humano (20,35,36), pero en las primeras décadas los resultados no fueron concluyentes debido a la tecnología de la época. Con el avance de la ciencia y el desarrollo de técnicas moleculares, celulares e inmunológicas utilizadas para la detección viral, ha sido posible aportar nuevas evidencias que llevan a la reflexión de la importancia que tiene este virus, el cual ha pasado desapercibido por muchos años tanto para la salud humana como en la salud animal.

A pesar de tener resultados negativos de la presencia viral en humanos, se han continuado estudios enfocados en análisis epidemiológicos con el fin de identificar si existe una asociación entre la presencia del virus y el desarrollo del cáncer de mama, en los cuales se han encontrado marcadores

biológicos virales en el tejido mamario y en la sangre de mujeres con y sin cáncer de mama en diferentes regiones geográficas, presentando una asociación significativa con la enfermedad (37–41). Adicional a esto, fue posible demostrar una relación de temporalidad entre la presencia del provirus y el cáncer debido a la detección de segmentos génicos provirales del VLB en muestras pareadas de tejido mamario de mujeres australianas obtenidas antes y después del diagnóstico de cáncer de mama, con al menos 10 años previos al desarrollo de malignidad, presentando una asociación significativa con la enfermedad (42). Los hallazgos recientes de la presencia del VLB en el humano lo proponen como un factor de riesgo para el desarrollo del cáncer de mama donde podría estar implicado en los procesos iniciales de transformación celular (43–45). En otros estudios el virus ha sido detectado en tejido pulmonar canceroso de humanos (46) en donde se han evidenciado alteraciones de rutas metabólicas asociadas a procesos de regulación del ciclo celular (47).

Si bien aún se requieren más estudios del impacto de este virus en los humanos, así como clarificar su vía de transmisión, diseminación y patogénesis en este hospedero, hasta el momento con la evidencia existente se plantea que la presencia del VLB en el humano podría considerarse como una potencial infección de tipo zoonótica (43,45). A pesar que hasta el momento aún existen muchos vacíos en el conocimiento con respecto a la presencia del virus en el humano, no se pueden ignorar los resultados de varios grupos de investigación, que dan pie a esta hipótesis (44). La mayoría de las infecciones zoonóticas conllevan a la aparición de enfermedades emergentes en los humanos mediadas por el aumento del contacto de las interfaces humano-animal (48), generando patrones de diseminación y cambios en la ecología microbiana, poniendo así en riesgo tanto la salud humana, como la animal.

Teniendo en cuenta el impacto que tiene este virus tanto en la salud humana como en la salud animal, en este documento se presentan los resultados obtenidos durante la ejecución de esta tesis doctoral, la cual fue realizada en el marco del proyecto “Búsqueda y Relación Filogenética del Virus de la Leucosis Bovina (VLB) en tejido Mamario Humano con y sin neoplasia, y en linfocitos de bovinos seropositivos” financiado por el Departamento Administrativo de Ciencia, Tecnología e Innovación de Colombia (Colciencias - actual Minciencias) y liderado por la Dra. Maria Fernanda Gutiérrez en la temática de la asociación del virus con cáncer de mama en mujeres

colombianas y evidencia del potencial zoonótico del virus. Estos resultados fueron obtenidos en el grupo de investigación de Enfermedades Infecciosas, en la línea de la epidemiología molecular de infecciones virales, con el apoyo del grupo de trabajo del Laboratorio de Virología de la Pontificia Universidad Javeriana, en colaboración con la Fundación Instituto de Inmunología de Colombia (FIDIC) y con la co-dirección del Dr. Manuel Alfonso Patarroyo.

Por tanto, para el desarrollo de esta tesis doctoral se planteó como objetivo general: *determinar la presencia del VLB circulante en Colombia, su asociación con el cáncer de mama y aportar evidencia sobre su potencial zoonótico*, al cual se le dará respuesta en el transcurso del documento organizado por capítulos correspondientes a cada uno de los objetivos específicos soportados con los artículos científicos publicados en revistas de alto impacto internacional. Finalmente, se encontrarán las conclusiones globales de la investigación, junto con las perspectivas, recomendaciones y anexos.

2. PLANTEAMIENTO DEL PROBLEMA Y JUSTIFICACIÓN

El cáncer representa uno de los principales problemas de salud pública a nivel mundial, siendo responsable de aproximadamente 163.5 muertes por cada 100,000 habitantes al año. Se considera una de las diez causas de muerte más comunes junto con otras enfermedades frecuentes como afectaciones cardiacas, respiratorias y accidentes cerebrovasculares (49); y se relaciona con una menor esperanza de vida tanto en hombres como en mujeres (50,51). En las mujeres, el cáncer de mama y de cuello uterino siguen aumentando de forma independiente a los índices de desarrollo humano (condiciones socioculturales y económicas), aunque las tasas de mortalidad han disminuido en los países de altos ingresos (52). En América Latina se ha registrado un rápido incremento en el número de casos dando como resultado un aumento de cáncer de mama en los últimos diez años (53,54). En Colombia, el cáncer de mama es el más frecuente en las mujeres, con más de 10,000 nuevos casos reportados cada año, con una incidencia ajustada por edad de 35.7 y una tasa de mortalidad de 12.9 por cada 100,000 habitantes para el 2020 (55).

El cáncer es considerado una enfermedad multifactorial, lo cual implica que el acúmulo de diferentes factores de riesgo aumenta la probabilidad de desarrollar la enfermedad (56). Dentro de los factores de riesgo descritos en la literatura para el desarrollo del cáncer existen factores tanto propios del individuo como factores externos incluyendo el estilo de vida de las personas, factores genéticos, comorbilidades, estados hormonales, exposiciones a radiación, exposición a sustancias químicas en los alimentos, contaminación ambiental y exposición a microorganismos con potencial oncogénico (57).

Históricamente los virus han tenido un impacto importante en el desarrollo de diferentes tipos de cáncer y se han propuesto como factores exógenos e iniciadores de los procesos de transformación celular y del desarrollo de la enfermedad (58). Aproximadamente del 15 al 20% de los casos de cáncer tanto en animales como en humanos se asocian con infecciones virales considerándolas como potenciales factores de riesgo e incluso en algunos casos ya se ha atribuido una relación de causalidad (59). Para los humanos, virus como el virus de la hepatitis B (VHB), el virus de la hepatitis C (VHC), el virus de Epstein-Barr (VEB), el virus del papiloma humano (VPH), el virus linfotrópico de células T humano tipo 1 (HTLV-1) y el virus del herpes humano tipo 8 (VHH-8)

han sido arduamente estudiados con respecto a su asociación con el cáncer, demostrando en su mayoría una relación de causalidad con diferentes tipos de cáncer (60).

Para el cáncer de mama, si bien aún no existe evidencia concluyente con respecto a la presencia de agentes virales que puedan tener un papel de causalidad con este tipo de cáncer, se han generado estudios desde la epidemiología donde se han encontrado diferentes agentes virales que han sido propuestos como factores de riesgo asociados al desarrollo del cáncer y como potenciales iniciadores de los procesos de transformación celular, incluyendo al VLB (61,62). Partiendo de la evidencia previa de la presencia del VLB en los humanos (31,32,35), se han planteado estudios de casos y controles realizados en EE.UU. (38,41), Brasil (39) y Australia (42) en los cuales se propone al VLB como un factor de riesgo intermedio asociado al desarrollo del cáncer de mama debido a los valores de Odd Ratios (OR) reportados que oscilan entre 2.7 y 5.0 con valores de *p* significativos, teniendo en cuenta los parámetros de referencia de factores de riesgo para el cáncer de mama (OR/RR 2.0-4.0) (63). Sin embargo, estos resultados aún no son concluyentes para considerar al virus como un agente causal ya que otros estudios han reportado resultados contradictorios, al no detectar la presencia del virus en mujeres con cáncer (64,65).

Teniendo en cuenta que aún existen dudas respecto al papel del VLB en humanos y su relación con el cáncer de mama, es necesario realizar más estudios enfocados en entender el rol que pueda tener el VLB en el humano y su asociación con el cáncer de mama; esto dado que antes de establecer un rol de causalidad de un patógeno con una enfermedad específica basados en los criterios de Bradford-Hill (66,67), es necesario realizar estudios epidemiológicos de análisis de riesgo que sean consistentes en poblaciones de diferentes regiones geográficas en donde se demuestre dicha asociación con la enfermedad, como evidencia para futuros estudios enfocados a la causalidad.

Como antecedentes de nuestro grupo de investigación en la Pontificia Universidad Javeriana, se había detectado la presencia del virus a través de sus proteínas (34) y de segmentos génicos confirmados por secuenciación (31) en mujeres con y sin cáncer de mama como evidencia de infección del virus en mujeres colombianas, sin embargo, con estos resultados previos no fue

posible establecer si existía una asociación directa con el cáncer, lo que llevó al planteamiento de esta tesis doctoral.

Previo al desarrollo de esta investigación no era claro el contexto actual del VLB circulante en Colombia ya que eran pocos los estudios de prevalencia del virus en el ganado colombiano y, por ende, no se tenía claro el riesgo de exposición que los humanos pudiesen tener a las fuentes de infección y de contagio. De este modo, surge el interés de profundizar en el conocimiento del virus con respecto a su situación actual en Colombia, su relación con el humano y su potencial vía de transmisión desde los bovinos, buscando proponerlo como una infección zoonótica. Para lograrlo, el desarrollo de la investigación se abordó desde dos enfoques: el primero buscando entender si el virus podría estar asociado con el cáncer de mama en una población de mujeres colombianas a través de un estudio de casos y controles, y considerarlo como un factor de riesgo para el desarrollo del cáncer; y el otro para entender el curso de la infección natural del virus en Colombia en el sector pecuario y en las interfaces con el humano, a través de la identificación de marcadores moleculares (segmentos génicos) obtenidos de mujeres sanas, bovinos, otras especies animales (búfalos y ovejas) y alimentos derivados de bovinos como la leche y la carne . La presencia de marcadores moleculares compartidos entre especies como evidencia de la infección y circulación del virus aportan al conocimiento de sus procesos de diseminación y transmisión tanto en granjas como en las interfaces con el humano.

Por último, se buscó evidencia experimental para confirmar la capacidad de infección del virus en líneas celulares humanas y la identificación del receptor celular que le permite al virus ingresar a su hospedero natural y eventualmente al humano, todo esto como soporte de la hipótesis de la capacidad zoonótica del virus.

3. OBJETIVOS

3.1. Objetivo General

Caracterizar la presencia del VLB circulante en Colombia, su asociación con el cáncer de mama y aportar evidencia sobre su potencial zoonótico.

3.2. Objetivos específicos

1. Caracterizar el VLB circulante en Colombia en muestras provenientes de mujeres sanas y en bovinos.
2. Identificar marcadores moleculares del virus en tejido y en sangre de mujeres colombianas con formación de tumor benigno y maligno en la mama como evidencia de la infección viral, y establecer su asociación con el cáncer de mama en la población estudiada.
3. Identificar y analizar marcadores moleculares de la infección por VLB en muestras obtenidas de especies distintas al bovino y en alimentos dispuestos para consumo humano, contemplados como potenciales vías de diseminación de la infección viral.
4. Identificar factores de riesgo asociados a la adquisición del virus en mujeres colombianas.
5. Aportar evidencia experimental de la capacidad zoonótica del virus a través de infección *in vitro* en líneas celulares humanas y análisis de su receptor celular.

4. CARACTERIZACIÓN DEL VLB EN COLOMBIA

Objetivo específico:

1. Caracterizar el VLB circulante en Colombia en muestras provenientes de mujeres sanas y en bovinos.

Uno de los principios de zoonosis se relaciona con la caracterización de los agentes patógenos en sus hospederos naturales, la presencia del mismo en hospederos accidentales y su capacidad de dispersión en el entorno (68). Para verificar este comportamiento, es importante conocer aquellas circunstancias epidemiológicas que favorecen el contagio con estos patógenos, así como sus perfiles de circulación y propagación en la naturaleza, para así entender su probabilidad de transmisión y diseminación, tanto entre especies animales, como en el humano; de este modo, es posible plantear estrategias integradas para la prevención y control (69).

En esta sección se presentan los resultados de un estudio de prevalencia en ganado bovino en las principales regiones ganaderas del país y la presencia del virus en mujeres sin patología de mama como evidencia de la circulación e infección del virus en el humano incluso antes de su asociación con alguna enfermedad.

4.1. Presencia del VLB en ganado bovino colombiano

Para poder tener un punto de partida de la presencia del virus en el humano fue necesario conocer la distribución actual del VLB en Colombia en su hospedero natural y en su entorno. En el 2013, Benavides y colaboradores reportaron una seroprevalencia del 19% en el ganado bovino en Pasto (70), y más adelante, Ortiz y colaboradores en el 2014 reportaron una seroprevalencia del VLB en el ganado bovino colombiano en las principales regiones ganaderas del país del 42% (71). Posterior a esto, Úsuga y colaboradores realizaron un estudio en las principales zonas lecheras de Antioquia en el cual reportaron una prevalencia del VLB del 44% a partir de la detección de un fragmento del gen *env* del virus (72).

Teniendo en cuenta estos antecedentes del virus en Colombia, como parte de una investigación conjunta con VECOL y AGROSAVIA, en el laboratorio de Virología se realizó un análisis de prevalencia por medio de la detección de segmentos génicos del virus para avanzar en el conocimiento actual de la distribución del VLB en el país en las principales zonas ganaderas (**Anexo 1**). Para esto se tomaron muestras de sangre periférica de 289 bovinos provenientes de 7 departamentos del país donde se encuentra la mayor actividad ganadera a las cuales se les realizaron análisis por PCR anidada para la detección de segmentos génicos virales. Las muestras positivas fueron confirmadas por secuenciación para determinar el genotipo circulante en Colombia. Se encontró una prevalencia del virus del 62% en las muestras de los bovinos analizados, con los genotipos G1 y G6 circulantes en Colombia.

Estos resultados aportaron a la epidemiología actual del VLB y resaltan la importancia de realizar seguimiento en el ganado bovino debido a la tasa de prevalencia encontrada, que comparado con estudios anteriores ha aumentado en los últimos años, similar a la situación de Latinoamérica (9). A pesar que el virus es de notificación obligatoria según las listas de control del ICA, el virus no hace parte de los microorganismos de control oficial para la libre circulación del ganado en el país, lo que dificulta implementar estrategias adecuadas de prevención y control (73,74). Al no formar parte de las infecciones de notificación obligatoria y de control oficial, no existe un seguimiento constante de la presencia del virus en los hatos ganaderos.

4.2. Presencia del VLB en mujeres sanas

Las infecciones zoonóticas se describen como infecciones y/o enfermedades transmitidas entre los animales y los humanos que pueden llegar a desarrollar brotes epidémicos e incluso manifestación de enfermedad en el humano. Por esto es importante analizar las interfaces que se tienen entre humanos y animales como indicadores de las potenciales vías de diseminación y dispersión entre especies, favoreciendo la adaptación y establecimiento en diferentes nichos ecológicos donde los patógenos puedan habitar y reproducirse (75). Como parte del avance en el conocimiento del virus, se realizó un análisis descriptivo de tamizaje poblacional para identificar si el virus se

encontraba presente en las mujeres incluso antes de desarrollar algún tipo de patología de la mama, tomando como base el antecedente de nuestro grupo de investigación del 2013 (31). De este modo, se analizaron muestras de tejido mamario sin desarrollo de ningún tipo de tumor como evidencia de infección del virus en muestras provenientes de mujeres sanas.

Se tomaron muestras de tejido mamario provenientes de cadáveres de mujeres con una causa de muerte distinta a cualquier tipo de cáncer y que no tuviera ningún tipo de alteración de la mama. La recolección de las muestras se realizó en la sede Central del Instituto Nacional de Medicina Legal (Bogotá), en colaboración con los médicos forenses entre marzo/2015 y noviembre/2016. Se incluyeron mujeres de 18 a 85 años, cuyos familiares hayan firmado el consentimiento informado para participar en el estudio. Se tomaron en total 145 muestras de tejido mamario, las cuales fueron enviadas a patología al Hospital San Ignacio para verificar que no existiera patología de la mama, según la guía de clasificación de patología mamaria de la Organización Mundial de la Salud (76). Como criterio de exclusión se contempló descartar aquellas muestras que no tuvieran una adecuada disponibilidad de tejido biológico, con alto contenido de grasa en la mama, o que al momento del análisis histopatológico se hubiera encontrado patología de la mama que presentara riesgo de cáncer, o diagnóstico de cáncer oculto al momento de la toma de las muestras.

La detección del provirus se realizó por medio de PCR anidada dirigida a un fragmento del gen *gag* posterior a la extracción de DNA total a partir de una fracción del tejido mamario fresco. Asimismo, como prueba complementaria se utilizó la PCR *in situ* dirigida a una región del gen *tax*, para visualizar las células que se encontraban infectadas con el virus directamente en el tejido y determinar si el provirus se encontraba en células de la glándula mamaria. Las muestras positivas por PCR líquida fueron enviadas a secuenciar para futuros análisis filogenéticos.*

* La metodología para la detección del provirus es equivalente a la registrada en el artículo "*Risk factor for breast cancer development under exposure to bovine leukemia virus in Colombian women: A case-control study*" que se encuentra en la siguiente sección del documento.

Para la población de estudio se registró una edad promedio de 44 años ± DS de 18.3. Las muestras de tejido mamario se clasificaron en 4 subgrupos posterior a los análisis de patología: enfermedad fibroquística de la mama (n=11), hiperplasia sin atipia (n=4), tejido normal (n=80) y fibrosis leve (n=50). Estas categorías corresponden a cambios normales en el tejido mamario y ninguna de estas implica un riesgo para desarrollo de cáncer (77). En la figura 1 se observan los resultados de la presencia del provirus distribuido en estas categorías para la población analizada. Del total de las muestras, el 71% de estas fueron positivas para la presencia del provirus (n=103), los resultados no fueron significativos para los grupos analizados. En la figura 2 se observa una imagen representativa de la PCR *in situ* para una muestra positiva para el gen *tax* del VLB.

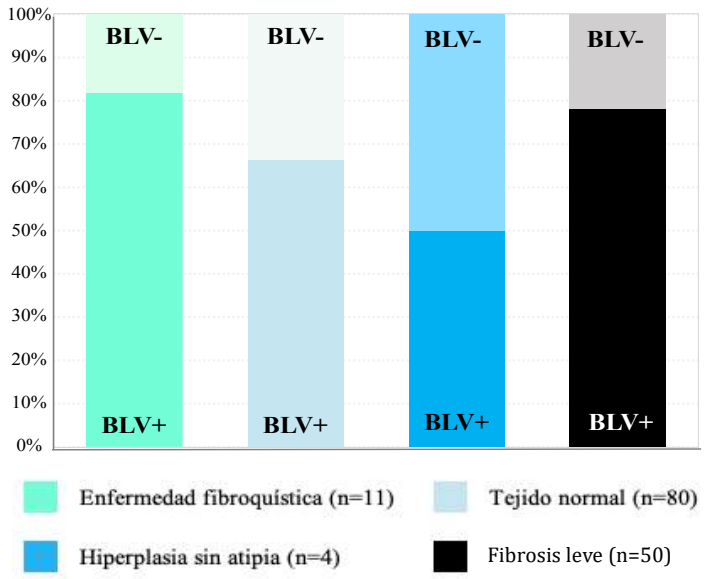

Figura 1. Presencia del VLB en estadio proviral en tejido mamario obtenido de cadáveres de mujeres colombianas sin presencia de patología de la mama, con causa de muerte distinta a cáncer. Se observan las propiedades histológicas de los tejidos analizados. Para cada diagnóstico se indica el n total. Los resultados se expresan en porcentajes (%) con respecto al n en cada grupo.

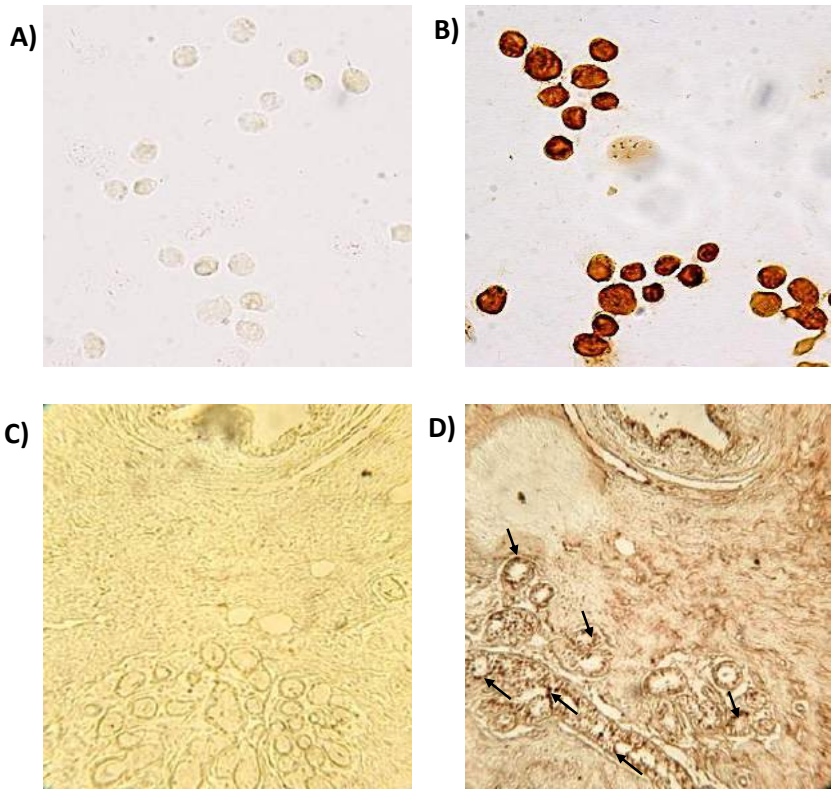

Figura 2. Detección del provirus de VLB por PCR *in situ* dirigido a un segmento del gen *tax*. Imagen representativa del diagnóstico por PCR *in situ* utilizando nucleótidos marcados con digoxigenina para la visualización de la amplificación de la PCR. **(a)** y **(b)** Corresponden a células FLK (Fetal Lamb Kidney), persistentemente infectadas con el VLB, utilizadas como control positivo. **(c)** y **(d)** Corresponden a una imagen representativa de tejido mamario positivo para el provirus. **(a)** y **(c)** son tejidos/células adyacentes a la reacción de PCR utilizadas como control negativo de reacción (sin cebadores ni Taq polimerasa), para verificar la especificidad de los anticuerpos utilizados. **(b)** y **(d)** representan las mismas muestras con reacción completa de PCR, revelado con DAB (diaminobencidina) con anticuerpos dirigidos a la digoxigenina. Las flechas en **(d)** indican la presencia del segmento de *tax* amplificado en células de la glándula mamaria (lóbulos) por su coloración café. Las imágenes fueron visualizadas en microscopio óptico Nikon Eclipse E200 a 10X. El ajuste de color fue el mismo para todas las imágenes.

Con estos resultados se pudo determinar la presencia del provirus en muestras de tejido mamario sano en una población de mujeres colombianas sin patología tumoral en la mama. La presencia del provirus en tejidos sanos sugieren la circulación del virus en un entorno natural, el cual logra establecerse en el humano incluso antes del desarrollo de enfermedad en la mama Estos resultados concuerdan con otros estudios donde plantean al virus como un factor iniciador en los procesos de desarrollo del cáncer y atribuyéndole una relación de temporalidad con el desarrollo del cáncer de mama (42,78).

La mayoría de las muestras incluidas en esta parte del estudio provenían de mujeres que vivían en Bogotá. En esta población no fue posible hacer un seguimiento detallado de la encuesta epidemiológica utilizada debido al desconocimiento de los familiares lo que llevó a datos faltantes para el seguimiento epidemiológico, sin embargo, en pocos casos se informó la procedencia de una zona rural. La presencia del virus en población sana es la primera evidencia para plantear al virus como un potencial virus zoonótico, que pueda estar ingresando al humano ya sea por un contacto directo con los animales o a través del consumo de alimentos provenientes de bovinos lo cual se abordará más adelante en el documento (68). Con estos resultados de tamizaje poblacional, se pudo observar que la frecuencia encontrada de la presencia del provirus en las muestras provenientes de las mujeres evaluadas fue similar a la prevalencia encontrada en el ganado vacuno proveniente de diferentes regiones del país (62%) (anexo 1), lo cual nos permite inferir que el virus se encuentra circulando tanto en el ganado como en los humanos lo cual da una información de la situación actual del virus en Colombia. Al haber identificado que el virus se encuentra circulando en una alta proporción en esta población de mujeres provenientes de Bogotá, se planteó un estudio de casos y controles para determinar si el virus podría considerarse como un factor de riesgo para el desarrollo del cáncer de mama y se decidió evaluar la presencia del virus en productos alimenticios derivados de bovinos dispuestos para consumo humano, los cuales se describen en los siguientes capítulos del documento.

5. ESTUDIO DE CASOS Y CONTROLES EN MUJERES CON CÁNCER DE MAMA EN COLOMBIA: ASOCIACIÓN CON LA PRESENCIA PROVIRAL DEL VLB

Objetivos específicos:

2. Identificar marcadores moleculares del virus en tejido y en sangre de mujeres colombianas con formación de tumor benigno y maligno en la mama como evidencia de la infección viral, y establecer su asociación con el cáncer de mama en la población estudiada.

El cáncer representa altas tasas de morbilidad y mortalidad a nivel mundial las cuales han venido en aumento en las últimas décadas. Para el 2020 se reportaron aproximadamente 19 millones de casos nuevos a nivel mundial, de los cuáles 2.3 millones fueron casos de cáncer de mama, convirtiéndose en la primera causa de cáncer global (79). Si bien en la actualidad existen estrategias para prevenir la enfermedad como estilos de vida saludables, consumo de una dieta rica en antioxidantes, disminución en el consumo de tabaco y alcohol y prevención de la obesidad entre otras (80), aún existen muchos otros factores que no son controlables y que están relacionados con el aumento de las tasas de incidencia de los diferentes tipos de cáncer.

Para el cáncer de mama, entre los principales factores de riesgo descritos en la literatura se incluyen las mutaciones *BRCA1/2 (breast cancer 1/2 gene)* y *PALB2 (partner and localizer of BRCA2)*, antecedentes de lesiones premalignas en la mama (ej. carcinoma *in situ* y lesiones con atipia), exposición a radiaciones en las primeras etapas de la vida y antecedentes familiares de cáncer de ovario o de mama (63,81) los cuales se consideran como riesgos altos al encontrarse reportados valores de RR/OR (riesgo relativo/odd ratio) mayores a 5. Adicional a estos, existen otros factores de menor riesgo (RR 2-5) como la edad de la menarquia, del primer embarazo y de la menopausia, así como la nuliparidad, la densidad de la mama, el estilo de vida y el consumo de anticonceptivos hormonales, o terapia hormonal posmenopáusica (81). Sin embargo, debido al incremento de las tasas de incidencia del cáncer de mama en las últimas décadas se ha propuesto que otros factores externos puedan estar contribuyendo a ese aumento drástico de la enfermedad (82), considerando incluso la participación de agentes infecciosos como los virus como potenciales factores de riesgo involucrados con el desarrollo del cáncer (61).

Entre los agentes virales encontrados en el tejido mamario humano se ha reportado la presencia del virus de Epstein-Barr (EBV), el virus del tumor murino mamario (MMTV), el virus del papiloma humano (VPH) y el virus de la leucosis bovina (VLB) (83), los cuales se han asociado con el desarrollo del cáncer de mama (61,84), lo que ha llevado a plantear la hipótesis de que estos virus podrían estar implicados en el proceso de la tumorigénesis y en la transformación inicial del tejido mamario, a pesar de aún no tener evidencia concluyente con respecto a un rol de causalidad en el desarrollo del cáncer de mama.

La carcinogénesis asociada a virus se describe en la literatura como un proceso lento que puede tardar décadas después de la infección inicial debido a que las infecciones virales pueden permanecer latentes o con una carga viral muy baja en el hospedero durante varios años hasta el desarrollo del cáncer, o incluso, existen individuos infectados que nunca desarrollan el cáncer. Es importante resaltar que existen diferentes mecanismos de acción según cada tipo de virus asociado con los procesos de oncogénesis (58,85,86). Algunos de ellos incluyen la modificación de factores epigenéticos y genómicos, como la acumulación de mutaciones, la inhibición de los mecanismos de reparación del ADN, la inducción de la inestabilidad del genoma del hospedero, la degradación de p53 en las células del hospedero y los procesos inflamatorios crónicos (87).

En la tabla 1 se encuentran los resultados previos reportados en la literatura en orden cronológico con respecto a la presencia del VLB en el humano, así como los estudios de casos y controles que reportan al virus como potencial factor de riesgo para el desarrollo del cáncer de mama.

TABLA 1. ESTUDIOS PREVIOS DE LA PRESENCIA DEL VLB EN MUJERES CON Y SIN CÁNCER DE MAMA.

Lugar de ejecución	Tipo de estudio	Tamaño de muestra	Presencia del VLB	OR/*p*	Año	Ref
Bogotá, Colombia	Descriptivo, presencia proteínas virales en tejido mamario con cáncer	n=56, tejido mamario con cáncer	Detección de proteína gp51 (7% de muestras)	NA	2006	Ochoa y col (34)
Bogotá, Colombia	Descriptivo, presencia en tejido mamario	n=53 c/u	36% CA 45% no CA	*p*= 0.32 Chi-cuadrado	2013	Mesa y col (31)
California, EEUU	Descriptivo, presencia en tejido mamario	n=219	44% de muestras de tejido mamario	NA Caracterización molecular de presencia viral	2014	Buehring y col (32)

California, EEUU	Casos y controles - cáncer de mama	n=114 c/u	59% CA 13% no CA	OR= 3.07, p= 0.0004, IC 1.66-5.69 Reg. Log. Multivar.	2015	Buehring y col (38)
Sydney, Australia	Presencia de virus antes y después de diagnóstico de cáncer	n=96, muestras pareadas	80% CA 41% no CA	OR= 4.72, p=0.003, IC 1.72-13.05 Reg. Log. Multivar	2017	Buehring y col (42)
Tandil, Argentina	Descriptivo, biología de tumor	n=85	23% CA	Relación con marcadores tumorales p= 0.009/0.044, Bivariado, Chi2 test	2018	Lendez y col (37)
Passo Fundo, Brasil	Casos y controles	n=72 c/u	30.5% CA 14% no CA	OR=2.73, p= 0.027, IC 1.18-6.29 Reg. Log. Multinomial	2019	Schwingel y col (39)
Irán	Descriptivo, presencia en tejido mamario y sangre	n=200 tejido mamario, 200 sangre	30% tejido 16% sangre	t-student p<0.05	2019	Kalillian y col (33)
Minas Gerais, Brasil	Descriptivo, población con consumo alto de leche cruda	n=88	95.9% CA 59% no CA	Relación con ocurrencia de cáncer Chi-cuadrado/ Test exacto de Fisher p < 0.001, OR=15.8237	2020	Delarmelina y col (40)

Estos estudios han permitido avanzar en el conocimiento del virus en el humano, en los cuales han sido consistentes los resultados en diferentes poblaciones con respecto a la propuesta del VLB como un factor de riesgo para el cáncer de mama. Teniendo en cuenta estos antecedentes, para el desarrollo de esta investigación se planteó un estudio de casos y controles no pareado para evaluar la asociación del virus con el cáncer de mama en una población de mujeres colombianas para una cohorte de pacientes beneficiadas del servicio de cirugía de mama en el Hospital de Méderi en Bogotá entre el 2016 y 2018 (Artículo 1). Para el análisis de riesgo se realizó una regresión logística multinomial ajustada por edad, y controlada con otros factores de riesgo del cáncer de mama descritos previamente en la literatura (63). Con los resultados de este estudio se obtuvieron diferencias estadísticamente significativas entre los grupos de casos y controles (p<0.031), apoyando la hipótesis de la asociación del VLB con el cáncer de mama, al obtener un OR ajustado = 2.450 (IC 95%:1.088–5.517), lo que lo propone como un factor de riesgo intermedio para la población analizada (63), en congruencia con lo reportado en otras regiones del mundo.

Considerando el cáncer como una enfermedad multifactorial, la identificación de factores externos como los virus que puedan estar asociados al desarrollo del cáncer abre una alternativa para introducir estrategias de prevención y control para en un futuro poder reducir las tasas de

incidencia de la enfermedad (57). La determinación de factores de riesgo que sean consistentes en distintas poblaciones a nivel mundial son la base para la toma de decisiones con respecto a políticas públicas que puedan plantear adecuadamente las estrategias de prevención y control enfocadas al bien de la comunidad.

Art1: Olaya-Galán NN, Salas-Cárdenas SP, Rodriguez-Sarmiento JL, Ibáñez-Pinilla M, Monroy R, Corredor-Figueroa AP, et al. (2021) Risk factor for breast cancer development under exposure to bovine leukemia virus in Colombian women: A case-control study. PLoS ONE 16(9): e0257492. https://doi.org/10.1371/journal.pone.0257492

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RESEARCH ARTICLE

Risk factor for breast cancer development under exposure to bovine leukemia virus in Colombian women: A case-control study

Nury N. Olaya-Galán[1,2]*, Sandra P. Salas-Cárdenas[2], Jorge L. Rodriguez-Sarmiento[3], Milcíades Ibáñez-Pinilla[4], Ricardo Monroy[4], Adriana P. Corredor-Figueroa[2], Wilson Rubiano[4], Jairo de la Peña[4], HuaMin Shen[5], Gertrude C. Buehring[5], Manuel A. Patarroyo[6,7,8], Maria F. Gutierrez[2]

1 PhD Program in Biomedical and Biological Sciences, Universidad del Rosario, Bogotá, Colombia, **2** Grupo de Enfermedades Infecciosas, Laboratorio de Virología, Departamento de Microbiología, Pontificia Universidad Javeriana, Bogotá, Colombia, **3** Department of Pathology, Hospital Universitario San Ignacio - Pontificia Universidad Javeriana, Bogotá, Colombia, **4** Hospital Universitario Mayor Méderi – Universidad del Rosario, Bogotá, Colombia, **5** School of Public Health, University of California, Berkeley, California, United States of America, **6** Molecular Biology and Immunology Department, Fundación Instituto de Inmunología de Colombia (FIDIC), Bogotá, Colombia, **7** Microbiology Department, Faculty of Medicine, Universidad Nacional de Colombia, Bogotá, Colombia, **8** Health Sciences Division, Main Campus, Universidad Santo Tomás, Bogotá, Colombia

* nury.olaya@urosario.edu.co

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Abstract

Viruses have been implicated in cancer development in both humans and animals. The role of viruses in cancer is typically to initiate cellular transformation through cellular DNA damage, although specific mechanisms remain unknown. Silent and long-term viral infections need to be present, in order to initiate cancer disease. In efforts to establish a causative role of viruses, first is needed to demonstrate the strength and consistency of associations in different populations. The aim of this study was to determine the association of bovine leukemia virus (BLV), a causative agent of leukemia in cattle, with breast cancer and its biomarkers used as prognosis of the severity of the disease (Ki67, HER2, hormonal receptors) in Colombian women. An unmatched, observational case–control study was conducted among women undergoing breast surgery between 2016–2018. Malignant samples (n = 75) were considered as cases and benign samples (n = 83) as controls. Nested-liquid PCR, *in-situ* PCR and immunohistochemistry were used for viral detection in blood and breast tissues. For the risk assessment, only BLV positive samples from breast tissues were included in the analysis. BLV was higher in cases group (61.3%) compared with controls (48.2%), with a statistically significant association between the virus and breast cancer in the unconditional logistic regression (adjusted-OR = 2.450,95%CI:1.088–5.517, p = 0.031). In this study, BLV was found in both blood and breast tissues of participants and an association between breast cancer and the virus was confirmed in Colombia, as an intermediate risk factor.

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1 Introduction

Cancer represents one of the greatest threats to public health worldwide. It is responsible for about 163.5 deaths per 100,000 inhabitants per year and is considered one of the most common causes of death, that is highly related with a lower life expectancy. In females, breast and cervical cancers are the two most commonly etiologies worldwide [1, 2]. Although high incidence rates have decreased in high-income countries, it continues to increase in other regions such as South America, with a rapid burden of the disease that has led to a peak of breast cancer in the last ten years [3, 4]. In Colombia, breast cancer is the main cancer etiology among women, with 13,380 new cases reported in 2018 and a mortality rate of 12.0 per 100,000 inhabitants [5].

Viruses have been proposed in the literature as potential starters of cellular transformation and tumorigenesis in both humans and animals [6, 7]. About 15–20% of cancers are correlated with a virus infection that leads to cellular transformation and tumorigenesis processes, with different mechanisms reported among them that are involved in the initial stages of cancer development [8]. Human papilloma virus, human herpes virus 8, Epstein-Barr virus, and hepatitis B and C virus are well-known examples of viruses associated with cancer in humans. Tumorigenesis is usually described as a slow process, that could take decades after the initial infection for the final outcome, in which viral infections could remain quiescent, latent or at a very low viral load in the host for several years until cancer development [6, 9]. Some of the mechanisms associated with cancer development are involved in epigenetic and genomic factors, such as the accumulation of mutations, inhibition of DNA repair mechanisms, induction of host genome instability, degradation of p53 in host cells and chronic inflammatory processes [10, 11].

Considering cancer as a multifactorial disease, the identification of external factors that may be associated with the development of cancer, including viruses, opens an alternative to introduce prevention and control strategies to reduce the risk of cancer [12]. However, there are still gaps in the knowledge in terms of cancer causation. Bradford-Hill criteria support the theory of causation of external factors related with cancer and suggests causality [13] as in the case of HPV with cervical cancer when fulfilling stated criteria [14]. Thus, in order to establish if a particular virus could be considered as a causative agent of cancer, the first necessary step is to perform studies from the epidemiological point of view. These studies should be focused on the identification of viral agents in specific cancer cells, with consistency along different geographical regions and populations, allowing the research community to identify potential risk factors associated with cancer development.

Regarding breast cancer, some viral agents have been reported in previous studies as potential risk factors of the disease [15]. Among them, Epstein-Barr virus (EBV), human papillomavirus (HPV), mouse mammary tumor virus (MMTV) and bovine leukemia virus (BLV) have been identified in human breast tissues [16]. It is important to note that no conclusive evidence has yet been found with respect to the causative relationship between the viral agents and the breast cancer development. However, it has been hypothesized that those viruses could be present on the breast tissues could be involved in the initiation of tumorigenesis and tissue transformation [17].

BLV is an exogenous deltaretrovirus (*Retroviridae* family), the causative agent of chronic infections in cattle, leading to leukemia and/or lymphoma development in between 5–10% of infected animals [18]. Unlike other retroviruses, deltaretroviruses (e.g. HTLV, STLV and BLV) cause malignant transformation mainly through a multifaceted protein (Tax) involved in several processes of regulation of the host cell [19, 20]. Tumorigenesis mediated by these viruses

occurs several years after infection, in which the viruses remain integrated into the host cells, with no or low evidence of the viral infection and without clinical manifestations.

In previous studies, BLV biomarkers (e.g. genes' fragments, antibodies and proteins) have been reported to be present in women [21–25]. In addition, the association of BLV with breast cancer has been suggested in case-control studies performed in USA, Brazil and Australia with Odd Ratios (ORs) ranging between 2.7 and 5.0, with significant *p* values for each specific population, proposing BLV as an intermediate risk factor for breast cancer development [26–28]. However, those results are inconclusive in terms of considering the virus a causative agent of breast cancer, as other studies have reported contradictory findings [29, 30]. In Argentina, significant results were obtained in the comparison between the presence of the virus with breast cancer prognostic markers such as Ki67 (cell division marker) and HER-2 (epidermal growth factor), suggesting that BLV might be involved in severity and progression of the disease, favoring cellular proliferation [31]. In Brazil, analysis of the tumor markers compared with the presence of BLV was performed, although no statistical differences were identified [28].

Breast cancer profile is determined by the presence/absence of specific tumor markers located on the surface of the cells, such as hormonal receptors (i.e. estrogens (ER) and progesterone (PR)), overexpression of HER-2 protein, and cell division marker Ki67 [32]. Together, these are the basis for categorizing breast cancer in terms of severity, progression of the disease and are useful for personalized treatment alternatives depending on the hormonal and tumor markers profile, that represent more than 21 subtypes of breast cancer [33]. Luminal A is the most prevalent subtype, which includes patients with positive hormonal receptors but negative HER-2 and it is considered a cancer subtype with slow-growing rate and good prognosis for recovery. In contrast, triple negative cancer subtype (HER-2 (-), ER (-) and PR (-)) represents a worst prognosis in the patients, as no specific treatment is available for this type of cancer with a high-rate of cellular proliferation. Few studies are available in terms of comparing cancer subtypes with other exogenous risk factors as viruses, focused on cancer progression and severity [34].

Molecular epidemiology studies in human populations focused on assessing BLV as a risk factor for cancer development are essential for clarifying the role of this virus in breast cancer and other cancer types, considering the oncogenic potential of other deltaretroviruses such as HTLV. This study was consequently aimed at determining the association between BLV and breast cancer in Colombian patients, as well as its correlation with progression tumor markers and cancer subtypes. BLV was identified as an intermediate risk factor in the analyzed population in line with other regions around the world.

2 Materials and methods

2.1 Study design

An unmatched, observational case–control study was designed for determining the association of the presence of BLV with breast cancer in a population of Colombian women between 2016 and 2018. Participants were women with breast tumors, benefited from the breast surgical service at Méderi Hospital (MH) located in Bogotá, Colombia. Following the histopathological diagnosis (see below, section 2.3) and the clinical records of the patients, participants were divided into two groups (i.e., cases and controls).

Cases were defined as patients diagnosed with any type of breast cancer, whilst patients diagnosed with benign pathology of the breast were considered as the control group, which was used as a reference for further analyses. The study was approved by the ethics committee of Universidad del Rosario (UR) and Méderi Hospital (Record No. CEI-ABN026-000 241, 2016). All procedures were performed in accordance with the ethical standards of the

institution and with the 1964 Helsinki declaration and its later amendments (last revision 2013). All the participants voluntarily signed an informed consent prior to the surgical procedures. Data obtained during the study was used under confidentiality.

From the patients benefited with the surgical intervention, the inclusion criteria were: women patients over 18 years of age, with a minimum tumor size of 4mm and enough biological material for both pathology follow-up and BLV detection. As exclusion criteria, samples with high content of fat tissue or low quality for molecular biology were discarded. Other diseases were not considered as exclusion criteria.

Sample size was calculated with a post-hoc strategy for the obtained OR of 2.45, with a case: control relation of 75:83, showing a power of 80% (type II error– 20%) and a confidence interval of 95% (type I error– 5%). Sample size was not possible to determine in advance, due to the lack of previous evidence in 2016 in Latin America and Colombia. A single study was published, reporting the presence of BLV in Colombian women [22]. None of the participants withdrew from the study.

2.2 Study variables

For the analyses of the results, exposure factor (independent variable) was defined by the presence of molecular markers of BLV, which was a dichotomous nominal variable categorized as positive or negative regarding the results obtained from the molecular biology techniques. Pathologies of the breast were considered as the dependent dichotomous nominal variables, defined as cases when categorized as malignant breast tissues, and as controls for benign breast tissues.

In addition, sociodemographic characteristics were obtained from the patients: age, educational level, city of origin, occupation, family background of cancer and parity history. Also, the complete clinical records of the patients were available, from which biomarkers of breast cancer used for the prognosis of the disease were obtained (hormonal receptors, Ki67, HER2) and sociodemographic variables were confirmed.

As confounding variables were considered: age, parities, background of cancer in the family and educational level. Only age was considered as a quantitative variable and was recategorized in groups $\geq$50 and $<$50, regarding to the risk factor group for breast cancer [35]. Confounding variables were used to adjust the model in further analyses.

2.3 Data and samples collection

After the inform consent was signed, participants answered a survey prior surgery, in order to collect the information of the sociodemographic variables mentioned above. Samples were collected consecutively and sequentially between 2016 and 2018 from patients who were scheduled for breast surgery in MH. Blood and breast tissue were taken from each patient for the study. Fresh breast tissue was placed in new, empty, sterile flasks and immediately transported to the virology lab at Pontificia Universidad Javeriana (PUJ). Blood samples were drawn into EDTA anticoagulant tubes and were also taken to PUJ.

2.4 Samples' preparation and pathological diagnosis

Collected breast samples were used for both histopathological diagnosis and viral detection. One section of the tissue was formalin-fixed in 10% formalin buffer and embedded in paraffin (FFPE) for histopathological classification in the San Ignacio University Hospital (HUSI), following the World Health Organization (WHO) international standards [36], approved protocol by the College of American Pathologists (CAP) for invasive cancer resection [37].

Pathology results were confirmed with MH's clinical records, and cases and controls were identified.

The second tissue section, as well as blood samples, were used for gDNA extraction with a High Pure PCR Template Preparation Kit (Roche ®, Mannheim, Germany), following the manufacturer's instructions. From blood, mononuclear cells were recovered with Lymphosep reagent ® (MP, Solon, OH—USA). Extracted DNA was stored at -20ºC until use.

2.5 Bovine leukemia virus detection

2.5.1 Nested-liquid phase PCR (nPCR). Quality of the extracted DNA from blood and breast samples was validated by amplifying the human GAPDH housekeeping gene. GAPDH-positive samples were used in further analyses. BLV detection targeted BLV genome regions (*gag*, *LTR*, *tax* and *env*). Primers and PCR cycling conditions from a previous report [21] were used here, with slight adjustments to PCR cycle conditions. Roche's PCR Master Mix (Cat. No. 11636103001, Mannheim, Germany) and Promega's GoTaq polymerase (Madison, WI—USA) were used for detection. Two researchers confirmed the results separately (NOG at UC Berkeley and SSC at PUJ), with an accuracy of 90%. The results were visualized by gel electrophoresis on 1.5% agarose gels stained with ethidium bromide. DNA extracted from the FLK cell line (constitutively infected with BLV) was used as positive control. As a negative control of reaction, RNAse/DNAse free water–molecular grade was used in each experiment. As an internal control of the laboratory, DNA from MCF7 (human breast cancer) cell line, which is negative to BLV, was used for discarding contamination of the areas and is included randomly in the experiments to avoid the presence of false positive results. Also, for avoiding crossed contamination, separate hoods were used for master mix preparation, DNA samples addition, and positive control addition to the PCR reaction. Samples were considered positive when at least one of the virus's genes was amplified and confirmed by Sanger sequencing, to ensure that it was a BLV product.

2.5.2 Direct *in situ* PCR (IS PCR). Direct *in situ* PCR was used as secondary test for viral detection in FFPE breast tissue, as previously described [21]. Slight changes were performed to the PCR protocol. From the FFPE tissues, extra cuts were performed and were attached to SuperFrost slides (Thermo Fisher ™, Hayward–CA, USA), as suggested by Nuovo [38]. The technique was optimized by targeting a longer region of the *tax* gene (nt 7197–7570, F: CTTCGGGATCCATTACCTGA; R:GCTCGAAGGGGGAAAGTGAA, 373bp). After paraffin removal, tissues were digested with pepsin (2mg/mL) with 0.05mL 2N HCl. *In situ* PCR was performed with the digoxigenin-labelled uracil system (Roche, Mannheim, Germany) and AmpliTaq Gold DNA polymerase—hot-start (Applied Biosystems ™, Carlsbad–CA, USA). Reactions were detected by an anti-DIG monoclonal antibody (mAb) (Roche, Mannheim, Germany) and revealed with DAB (diaminobenizidine) solution, following the manufacturer's instructions (Vector ®, Burlingame–CA, USA). An adjacent tissue section from each sample, without Taq polymerase and without primers, was evaluated to verify that no cross-reaction or non-specific attachment occurred by the DIG-labelled uracil and/or by the mAb as a negative reaction control. FLK cell line smears were used as positive controls. Slides were observed under a Nikon Eclipse E200, at 10x/40x magnification. Samples were considered positive when a dark brown-red stain was visualized in the mammary epithelial cells (ducts and lobules), and were clearly differentiated from the background. Negative control tissue displayed no brown color.

2.5.3 Viral proteins detection by immunohistochemistry. Viral capsid protein (p24) was detected by immunohistochemistry (IHC) on an extra slide of breast tissue. Endogenous peroxidases were inactivated with 3% hydrogen peroxide in methanol solution, followed by

unmasking antigens in citrate buffer (10mM sodium citrate buffer, pH 6.0) in boiling water (95ºC) for 30 min. Tissues were blocked with 1.5% fetal horse serum (FHS) in PBS preventing non-specific antibody attachment. A mAb targeting p24 diluted 1/10 in blocking solution and a biotinylated horse anti-mouse IgG secondary antibody (1/200) (Vector Laboratories Cat# BP-2000, RRID:AB_2687893) were used for viral detection. An ABC kit (Vector ®, Burlingame–CA, USA) was used as reaction enhancer and the DAB reagent (Vector ®, USA) was used for peroxidase activity detection. Results were observed on a Nikon Eclipse E200 optical microscope at 10x/40x magnification. Dark brown coloring in mammary epithelial cells was considered positive, representing p24 in the cells. As a negative control, an adjacent tissue section was treated only with the secondary antibody.

For the statistical analyses for the risk assessment, presence of BLV in the breast tissues of the patients was considered positive when at least one of the PCR techniques (nested-liquid PCR or *in situ* PCR) was able to identify the viral DNA and was confirmed by sequencing. Correlation of the presence of the virus in breast and blood was also evaluated.

2.6 Cancer prognosis biomarkers and hormonal receptors

Information regarding the tumors' hormone profile (progesterone receptors–PR and estrogen receptors–ER) and prognostic markers (HER2 and Ki67) were retrieved from the cancer patients' clinical records. Tests were performed by the pathology diagnosis laboratory of Méderi Hospital, following internal protocols. Immunohistochemistry was performed by prediluted monoclonal antibodies directed to the specific markers from Dako/Agilent ® (Santa Clara—CA, USA). HER2 protein was detected with HercepTest (Cat.No.SK00121-5), rabbit anti-human monoclonal antibody (Agilent Cat# IR084, RRID:AB_2617140); progesterone receptors (PR) with mouse anti-human monoclonal antibody (Agilent Cat# IR06861, clone PgR636, RRID:AB_2890066); estrogen receptors (ER) with rabbit anti-human monoclonal antibody (Agilent Cat# IR084, clone EP1 RRID:AB_2617140) and Ki67 was detected with mouse anti-human monoclonal antibody (Agilent Cat#IR62661-2CN, cloneMIB-1, RRID: AB_2890068). Results were visualized on an optical microscope Olympus BX43 and results were reported as part of the findings given to the patient in the histopathological report. Tests were not performed to patients with pathologies other than breast cancer.

2.7 Principles of comparability, validity and reliability

Defined principles of comparability for analytical case-control studies were used to avoid bias and assure validity in our study [39–41]. For example, the selection of cases and controls form the same basis population, and the control of other risk factors described for breast cancer in the literature (e.g., age, nulliparity, family background of breast cancer and educational level) [33] in the multivariate analysis fulfilling the deconfounding principle and obtaining adjusted OR values.

All variables and samples were measured and processed identically, and by blinded investigators, avoiding measuring bias and having no differences in the manipulation between cases and controls samples guaranteeing the comparable accuracy principle. Classification bias of dependent variable (breast tissue histopathological diagnosis) was controlled by following the CAP protocol for breast cancer diagnosis [37] and was verified by the clinical records at MH. For BLV detection, samples were determined as positive when at least one of the molecular techniques (nested-liquid PCR or *in situ* PCR) showed positive for viral DNA in the breast, as direct evidence of the presence of BLV in the tissue. PCR products were sequenced by Sanger technology confirming identity >95% with BLV.

2.8 Statistical analysis

SPSS (Ver. 25.0, IBM Corp., Amonk, NY, USA) and STATA (Ver. 15, StataCorp LP, College Station, TX, USA) were used for statistical analysis. Initially, a descriptive analysis was carried out to all the qualitative variables, from which frequencies and percentages were determined. Measurements of central tendency and dispersion (e.g., average, range, and standard deviation) were used for 'age', which was the only quantitative variable. Normality was measured by Shapiro Wilk and Kolmogorov Smirnov tests.

According to the expected values in contingency table (<5), Pearson chi-square or exact Fisher's test were used for comparing cases and controls variables, as well as cancer prognostic biomarkers (hormonal receptors, Ki67 and HER2) with the presence of the virus. The association between BLV and breast cancer was carried out by an unconditional multivariate logistic regression for the estimation of the Odd Ratios(OR) with a 95% of confidence interval (CI) adjusted by risk factors associated with breast cancer and other confounding variables identified in the study (i.e., age, parity, background of breast and ovarian cancer, educational level, occupation, and city of origin). The prediction area and its respective 95% CI were determined with the ROC curve. p values. Less than 0.05 were considered as significant for the study ($p < 0.05$) for all the statistical analyses). As a secondary analysis, pre-malignant samples diagnosed in the histopathological observations were included in the data set, and a multinomial logistic regression was carried out, due to the risk of these pathologies for breast cancer development in the future [42].

3 Results

3.1 Population's sociodemographic and biological characteristics

This cohort of patients, obtained between 2016 and 2018, was constituted by a total of 168 participants. After the histopathological classification, 75 patients were diagnosed with cancer (malignant tumors) and were included into the cases group; 83 patients were diagnosed with benign pathologies of the breast and were included in the control group. Ten of the patients were diagnosed with pre-malignant lesions of the breast (i.e., hyperplasia with atypia, *in situ* carcinoma). Those were excluded from the initial analysis, resulting in a definitive cohort of 158 patients.

Participants were aged between 18 and 89 years, and lived in Bogota city, where Méderi Hospital is located. Statistically significant differences were identified between cases and controls in terms of age, educational level, parities, and occupation in the bivariate analysis (See Table 1). In the cases group, patients were older compared with the control group. In addition, cases group had a lower educational level compared with the control group, as well as the occupations reported, which were more frequent to be on the home-basis in the cases group. Sociodemographic characteristics could be influenced by geographical regions and cultural behaviors. In the analyzed population, educational level and occupation were significant for the model, and were included in the multivariate analysis.

3.2 Histopathological classification and viral detection

Frequencies of the most relevant breast's pathologies for the cases and control groups were included in both groups. Malignant tumors (cases) were described as invasive ductal carcinoma, invasive lobular carcinoma, and other malignancies (i.e., malignant phyllodes tumor, sarcoma, mixed type carcinoma and invasive poorly differentiated carcinoma). Within the control group, benign pathologies were diagnosed as fibroadenomas, hyperplasia without atypia, papillary lesions, and others less frequent (i.e., simple cysts, benign phyllodes tumor,

Table 1. Comparison of sociodemographic and biological characteristics between malignant (cases) and benign samples (controls).

	Cases	Controls	
	Malignant (n = 75)	Benign (n = 83)	*P value*
	n (%)	n (%)	
Age			
$\bar{x} \pm SD$	66.15 ± 11.89	40.55 ± 18.01	<0.001
≥50	47 (62.7)	10 (12.0)	
Other characteristics			
Origin			0.047
Bogotá	61 (81.3)	56 (67.5)	
Other	14 (18.7)	27 (32.5)	
Family background of breast/ ovarian cancer	46 (63.9)	48 (58.5)	0.304
Parity	64 (86.5)	48 (58.5)	<0.001
Educational level			<0.001
Elementary school	31 (41.9)	11 (13.4)	
High School	26 (35.1)	28 (34.1)	
Vocational and professional studies	17 (23.0)	43 (52.4)	
Occupation			<0.001
Home-based activities	38 (50.7)	15 (18.1)	
Living/working in rural areas	1 (1.3)	1 (1.2)	
Other [a]	36 (48.0)	67 (80.7)	

[a] Other: industry, office, own business, marketing, etc.

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mastitis). Frequencies of each pathology are found on Table 2 and details of the complete data set can be found in S1 Table in S1 File.

Table 2 also shows results for the viral detection reported by each technique that was performed. Results are shown by the presence of the virus in blood and breast tissues, organized

Table 2. Histopathological diagnoses and viral detection.

Histopathological diagnoses	BLV DETECTION/TECHNIQUE §					BLV (+) ** *n (%)*
	Nested PCR		IS PCR *n (%)*	nPCR + IS PCR# *n (%)*	IHC (p24) *n (%)*	
	Breast tissue *n (%)*	Blood *n (%)*				
Cases (n = 75)						46 (61.3)
Invasive ductal carcinoma (n = 37)	15 (40.5)	13 (35.1)	9 (24.3)	3 (8.1)	4 (10.8)	
Invasive lobular carcinoma (n = 10)	6 (60.0)	5 (50.0)	5 (50.0)	3 (30.0)	2 (20.0)	
Other malignancies [a] (n = 28)	12 (42.9)	10 (38.5)	7 (25.0)	4 (14.3)	3 (10.7)	
Controls (n = 83)						40 (48.2)
Fibroadenoma (n = 43)	16 (37.2)	12 (28.6)	11 (25.6)	8 (18.6)	3 (6.9)	
Hyperplasia without atypia (n = 10)	2 (20.0)	1 (10.0)	1 (10.0)	1 (10.0)	1 (11.1)	
Papillary lesions (n = 11)	6 (54.5)	3 (27.3)	3 (27.3)	3 (27.3)	2 (22.2)	
Other benign tumors [b] (n = 19)	6 (31.6)	4 (22.2)	4 (21.16)	4 (21.1)	3 (10.0)	

[a]Other malignancies: malignant phyllodes tumor (n = 1), sarcoma (n = 1), mixed type carcinoma (lobular and ductal, n = 3), invasive poorly differentiated carcinoma (n = 20), mucinous carcinoma (n = 3).

[b]Other benign tumors: Simple cysts (n = 3), benign phyllodes tumor (n = 1), mastitis (n = 1), fibrocystic change (n = 7), sclerosis adenosis (n = 7).

§ Results are shown as frequencies per each diagnosis. Percentages were calculated for each specific diagnosis within cases and controls.

#nPCR+IS PCR indicate results for samples that were simultaneously positive for both techniques.

** BLV (+) represents the total amount of positive samples for cases and controls.

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by the cases and controls groups as well as for each specific histopathological diagnosis. Details of the complete data set could be found in the supplementary material. Each molecular technique was carried out for detecting different targets of the virus (i.e., viral genome segments and viral proteins). The use of different techniques contributes to the understanding of the biological implications of the virus and strengthens the validation of the diagnosis. Results considered for the risk assessment of the association of breast cancer with the presence of the virus, were those in which proviral DNA of BLV was found by the molecular techniques and confirmed by sequencing on the breast samples. S1 and S2 Figs in S1 File show results of nested and *in situ* PCR targeting *gag* and *tax* region respectively. Samples that showed positive to both techniques (nPCR + IS PCR, Table 2) indicated that more than one genetic region of the virus was identified in the same sample. Detection of BLV in the blood and immunohistochemistry were performed for a better understanding of the biological implications of the presence of the virus in human beings. BLV was detected both in blood and breast tissues with a correlation of 94% in the positive samples of the study. IHC results indicate the presence of viral proteins (p24) in the breast tissues. For this study, only 10% of the samples showed the presence of p24 proteins. No significant statistical differences were identified regarding BLV detection among the specific histopathological diagnosis of the cases and controls samples. Detection techniques were directed to the proviral stage of the virus, which remains for long terms in the host. No active viral infection was evaluated. Presence of p24 proteins suggest evidence of complete viral particles in the tissues, besides the evidence of its proviral genome.

3.3 Association between presence of BLV and breast cancer

BLV was found in 61.3% (n = 46) of patients with cancer (cases) and in 48.2% (n = 40) of the control group, being with a higher presence in the cases group. Results obtained in the unconditional logistic regression showed that presence of BLV was significantly associated with breast cancer outcome, compared with the benign pathologies of the breast (OR = 2.45, CI 95%: 1.088–5.517, $p = 0.031$, Table 3), after adjusting with confounding variables including age, parities, background of breast and ovarian cancer in the family, educational level, and occupation. The model prediction area using the ROC curve was significant, with 83.1% (95% CI 76.7%-89.5%, $p < 0.001$, Fig 1). ROC model showed a sensitivity of 77.1%, specificity of 71.6% and accuracy of 74.2%.

3.4 Complementary analysis for pre-malignant samples

As the association of BLV with breast cancer was identified in the cohort of patients, pre-malignant samples obtained in the histopathological diagnosis were also included for a secondary analysis. Some of the pre-malignant diagnoses in the breast are considered as precursory lesions of breast cancer, increasing the risk of cancer outcome (e.g. *in situ* carcinomas) [42].

In our study, ten of the patients of the initial cohort were diagnosed with pre-malignant lesions of the breast, distributed as follows: atypical hyperplasia (n = 3), *in situ* carcinoma (n = 4), papillary lesion with atypia (n = 2) and atypical phyllodes tumor (n = 1). These patients were aged between 24–81 years, with a mid-age of 59.60 ± 17.49 years. In terms of educational level, occupation and city of origin, patients were evenly distributed.

Although pre-malignant lesions were not considered initially in the study design, within the cohort of patients ten of them were confirmed as pre-malignant after surgery; and considering the risk of pre-malignant lesions to evolve into breast cancer, and the natural history of the disease, we evaluated if the presence of the virus also influenced the OR in patients with this diagnosis as a complement to the initial analysis, also in terms of not losing valuable information obtained from the study.

Table 3. Unconditional logistic regression adjusted by risk factors for breast cancer (age, parities, background of cancer in the family and educational level).

Variables	Malignant (n = 75)		
	ß	OR (95% CI)	*P value*
Viral presence			
BLV POS	0.902	2.450 (1.088–5.517)*	0.031*
BLV NEG	- -	1.00 (Reference)	- -
Age			
≥50	2.104	8.202 (3.163–21.270)	<0.001
<50	- -	1.00 (Reference)	- -
Nulliparity			
Yes	-0.714	0.490 (0.174–1.380)	0.177
No	- -	1.00 (Reference)	- -
Family background of breast/ovarian cancer			
Yes	0.140	1.151 (0.499–2.655)	0.742
No	- -	1.00 (Reference)	- -
Education level			0.06
Elementary school	1.155	3.176 (1.114–9.051)	0.031
High School	0.473	1.604 (0.611–4.209)	0.337
Vocational and Professional studies	- -	1.00 (Reference)	- -

* Significant results obtained for the presence of the virus in the breast cancer population (malignant–cases) compared with benign samples as the reference (control) group. <0.05 *p* values were considered statistically significant for the study.

OR-adjusted values.

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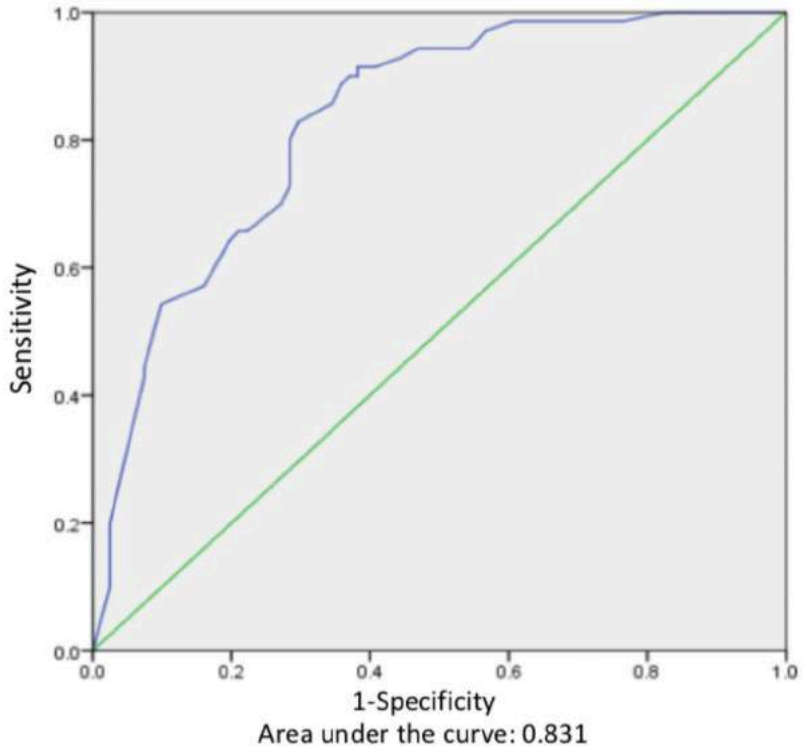

Fig 1. ROC curve of the presence of BLV model predictive of breast cancer.

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Table 4. Correlation between hormone receptors and prognostic markers for breast tumors with BLV presence.

TUMOR BIOLOGY	BLV PRESENCE		*P* value
	BLV POS n (%)	BLV NEG n (%)	
Progesterone Receptors—PR (n = 76)			0.373
POS (n = 59)	36 (61.0)	23 (39.0)	
NEG (n = 17)	9 (52.9)	8 (47.1)	
Estrogen Receptors—ER (n = 76)			
POS (n = 67)	42 (62.7)	25 (37.3)	0.094
NEG (n = 9)	3 (33.3)	6 (66.7)	
Cellular division marker—Ki67 (n = 68)*			
>14 (n = 39)	20 (51.3)	19 (48.7)	0.112
≤14 (n = 29)	20 (69.0)	9 (31.0)	
Epidermal growth factor—HER2 (n = 69)*			
POS (n = 15)	7 (46.7)	8 (53.3)	0.239
NEG (n = 54)	33 (61.1)	21 (38.9)	

*Tests were not performed to all the patients.

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A multinomial logistic regression was performed including the pre-malignant samples in the model; thus, breast cancer (cases, n = 75) and pre-malignant samples (n = 10) were compared with benign samples as the reference group. Model was also adjusted with the confounding variables, and a 95% of confidence interval.

In the multinomial logistic regression, an association between the presence of the virus and breast cancer was also identified (Adjusted OR 2.477, 95% CI 1.108–5.538, $p = 0.027$). However, presence of BLV in the pre-malignant lesions did not show a significant association, although OR value was greater than 1.0 (OR 1.133,95% CI: 0.286–4.488, p = 0.859).

3.5 Correlation between BLV presence and tumor prognostic markers

When comparing presence of BLV with breast cancer tumor prognostic markers (hormonal receptors, HER2 protein and Ki67), no statistically significant differences were found in the bivariate analysis. This analysis was carried out to determine if the virus could have any implications in the aggressiveness and prognosis of breast cancer. Although no significant differences were identified, a high percentage of BLV positive samples were also positive for estrogen (63%) and progesterone (61%) receptors (Table 4). Bivariate analysis was performed only to the malignant group (n = 75), compared with the viral presence, due to the use of those markers as prognostic markers of breast cancer and are not performed routinely to all the specimens.

4 Discussion

Viruses are considered potential initiators of cancerous diseases. Classically, HPV and HBV have been studied for their association with cancer in humans among other viruses as well [9]. However, investigating the role viruses could have in cancer development helps to advance in the current knowledge about cancer etiology. In the future, prevention and control strategies could be implemented to reduce the risk of exogenous risk factors leading to cancer diseases [43]. In this study, an association of the presence of BLV with breast cancer was identified with an OR = 2.45, CI 95%: 1.088–5.517 and a significant *p* value ($p = 0.031$). According to the American Cancer Society (ACS), this OR value represents an intermediate risk factor (ORs

2.0–4.0) for cancer development, along with other risk factors such as radiation, hormonal exposure and having a history of relatives suffering breast/ovarian cancer [33].

In previous studies, higher OR values were identified supporting the association between BLV and breast cancer in other regions like Australia (4.72 OR, 1.71–12.0 95%CI) [27] and the USA (3.07 OR, 1.66–5.69 95%CI) [26]. Nevertheless, differences between the obtained OR values with respect to the presence of the virus in humans could be influenced by sociodemographic conditions of the evaluated populations. The most relevant ones are their ethnic profiles, cultural behaviors, economical income and lifestyle habits, including food consumption. In contrast, the OR value obtained for Colombia was quite similar to that obtained in South Brazil (2.73 OR, 1.18–6.29 95%CI) [28]. Both populations have similar conditions in terms of sociodemographic characteristics and genetic history, involving native American, African and South European ancestry [44, 45]. On the other hand, USA and Australia have greater influence from Northern Europe and Asian countries [26, 46]. Taken together, viral presence and ethnicity could be factors involved in breast cancer outcomes [47]. Regarding the socioeconomical factors, conditions such as accessibility to health insurance, late diagnosis of the disease, resources for management of the disease and lifestyle could also affect these populations, causing higher rates of breast cancer as it has been shown in the literature [48, 49].

Additionally, considering that cattle are naturally infected by the virus, and high prevalence rates have been reported worldwide [50], another possibility of differences among the OR values in those regions could be related with the intake of cattle-derived food products. USA and Australia are classified by the FAO (Food and Agriculture Organization of the United Nations) as high meat and milk consumption countries, while Latin American countries are considered intermediate consumers [51, 52]. The presence of BLV DNA as a biomarker of viral presence in cattle-derived food products was recently reported in Colombia [53]. Therefore, in spite of having no evidence of infectious viral particles, the presence of viral DNA supports the hypothesis of transmission through consuming infected food products, probably through a cell-to-cell infection mechanism [54]. However, further studies are needed to fully understand the virus's transmission pathway to humans.

Previous studies in Colombia have shown evidence of the virus in women [22, 23], but have not determined whether BLV could be a risk factor for the Colombian population or if it has any implications in the progression of the disease. Results obtained in the current study support the hypothesis of BLV being associated with breast cancer as reported in other regions, but nosignificant difference between the presence of the virus, specific histopathological diagnosis nor the tumor prognosis markers were not found (Table 4). Results showed that BLV could be present in different profiles of the mammary epithelial cells, including tumor profiles and diagnoses. In contrast, results obtained in Argentina [31] showed correlation between BLV and prognosis markers in the breast tumors of women in Tandil.

Analyzing the relationship between breast cancer biomarkers (i.e., hormonal receptors, Ki67 and HER2) and the viral presence, it might give a background of the tumor microenvironment. Itcould be favoring an active viral transcription stage and a specific subtype of breast cancer that is probably associated with BLV infection. Previous *in vitro* studies have shown higher BLV transcription rates induced by progesterone and corticoids stimulation through LTR region activity [55]. In Argentina, a significant correlation between BLV and the Ki67 biomarker was found [31], suggesting that BLV might be involved in early stages of cancer development, as this biomarker indicates an active cell division and proliferation stage of breast cancer. Even if in our study non-significant results were obtained when comparing tumor markers with the presence of BLV, it is important to highlight that most of the samples that were positive for the virus were also positive for hormonal receptors (n = 36 for ER and n = 42 for PR). Moreover, most of the lobular cancer subtype samples (6 out of 10) were

positive to the virus. Even if no statistical correlation was possible to obtain due to a small amount of samples with this specific diagnosis, it is important to consider in the future studies of BLV analyses regarding the subclassification of breast cancer, in order to identify if it could be associated to specific subtypes.

Another key point in the current study, was the evidence of BLV in both breast tissues and blood of Colombian women with a concordance of 94%. BLV has been reported in breast tissues [21, 22] and blood [24, 25], but not in the same target population. Evidence of the virus in both blood and breast tissues from the same patient supports the hypothesis that blood might be helping to spread the virus throughout the body, until it reaches other tissues such as breast and lungs, in which the virus has been described to date [56]. Also, there is a possibility that the virus could be reaching other tissues as well, that have not yet been studied, and might interact with permissive cells mediated through the cellular receptors, which are proposed to be AP3D1 [57] or CAT1/SLC7A1 [58].

Now, taking into account the BLV biomarkers that were identified, it is important to underline that in this virus some fragments of its viral genome could be lost after infection [59, 60]. Therefore, revising the presence of the virus with different markers decreases the chances of false negative samples. Moreover, sequencing also guarantees that the amplified products belong to BLV and not to unspecific amplifications. It is important to highlight that the majority of the positive samples showed positive for at least two biomarkers, mainly for the detection in blood and breast (94% of concordance) (S1 Table in S1 File). PCR results indicate presence of the virus in proviral stage (integrated in the host cell genome), while IHC indicates presence of viral proteins, as evidence of active viral replication. In samples in which p24 was identified, other viral markers were also found as expected. Bearing in mind the biology of viruses involved in cancer development, previous evidence in the literature suggests that cancer manifestations could appear several years after initial infection and is not necessary to have an active viral cycle, with the production of new viral particles to induce cellular transformation processes [6].

One of the evaluated markers was the presence of a fragment of *Tax* region within the breast tissues in the *in-situ* PCR. Finding this biomarker, might be associated with cellular transformation, as it happens to cattle and humans in the leukemia development in the case of BLV and HTLV respectively [19, 61]. Tax protein is described as a multifaceted protein which has the capacity of co- regulate different cellular and viral pathways. It acts as a transactivator, inhibits mechanisms of DNA repair and also regulates proliferation and apoptosis pathways, even in few amounts of the protein [62]. However, it remains unclear the specific mechanism or role that BLV might have in humans, besides its association with breast cancer [26–28]. In our study, it was not possible to detect *tax* region with the *in situ* PCR in all of the positive samples, although sequences were confirmed for those cases targeted to *gag* region. Previous evidence in the literature reported for BLV and HTLV suggests that the viruses are not always integrated completely, with the evidence of genomic deletions in natural infection. Nevertheless, it is not clear the implications of these deletions to the viral cycle, as it has been found both in asymptomatic individuals, as well as in advanced stages of cancer disease [60, 63]. Further studies are needed to make clear the functionality of BLV in humans, as well as its integrations profiles to elucidate a plausible role for cancer outcome.

Besides the analysis performed for cases and controls, a secondary analysis involving the pre-malignant samples was considered. These samples were not intended to be in the initial design but were incidental findings of the study. We are conscious it was a few number of samples (n = 10), but considering the risk for cancer development with these lesions [42], it was interesting to observe if the virus could have any impact on these samples as well. In the multinomial logistic regression, the association between BLV and breast cancer was also confirmed

with an adjusted OR 2.477, 95% CI 1.108–5.538, $p = 0.027$. Whereas no significant results were obtained when analyzing the association between the presence of the virus and the pre-malignant samples. Further studies with this specific diagnosis are highly recommended to evaluate the impact of BLV in these samples and potential cancer outcome.

Prospective studies with human participants in cancer research are challenging. Obtaining matched-samples for case-control studies, in this case from malignant and benign tumors of the breast, takes long terms due to the availability of surgeries and interventions. In contrast, retrospective studies open the possibility of obtaining higher number of samples from archives, although the quality of the samples is not always indicated for molecular analysis and missing data from the participants is common. An advantage of collecting samples directly from the surgeries provides better quality of breast tissues for DNA processing and availability of blood, as well as the availability of the data collected directly from the participants, which enriches the epidemiological studies. In some cases, the complete clinical records are not available in archive samples.

In our study we performed a design with high complexity in the conception, as well as the detection of the virus through multiple techniques to guarantee processes of validity and comparability. Although paired samples were not taken, the multivariate analysis was controlled with confounding variables described in the literature and was adjusted by age. However, it is important to highlight that even if confusion for the analysis was reduced, it is possible to still have other variables that were not possible to control, leading to a residual confusion for the analysis. Nevertheless, statistical differences were obtained in the study between the cases and controls group, supporting the hypothesis of BLV being associated with breast cancer, contributing to the research field of the role of BLV in humans.

5 Conclusion

In conclusion, this study showed the association of BLV with breast cancer in the analyzed population, with an OR value similar to that obtained in Brazil. BLV could be considered as an intermediate risk factor for breast cancer, although further studies are needed to elucidate the role and mechanisms of the virus in humans. Evidence of BLV both in blood and breast tissues, suggests a possibility for early detection of the virus in screening studies.

This study is an incremental finding for the current situation of BLV in humans and its association with breast cancer. Prevention and control strategies of BLV in cattle could favor to stop the transmission of the virus to humans. Eradication programs worldwide should be considered, as it has already been done with eradication policies in Europe, Australia, and New Zealand.

Supporting information

S1 File. Representative results of nested and *in situ* PCR. Participants' histopathological diagnosis and viral detection.
(PDF)

S1 Raw images.
(PDF)

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Author Contributions

Conceptualization: Nury N. Olaya-Galán, Maria F. Gutierrez.

Data curation: Sandra P. Salas-Cárdenas, Jorge L. Rodriguez-Sarmiento, Milcíades Ibáñez-Pinilla, Ricardo Monroy, Wilson Rubiano, Jairo de la Peña, HuaMin Shen, Gertrude C. Buehring.

Formal analysis: Nury N. Olaya-Galán, Jorge L. Rodriguez-Sarmiento, Milcíades Ibáñez-Pinilla, Gertrude C. Buehring, Maria F. Gutierrez.

Funding acquisition: Maria F. Gutierrez.

Investigation: Nury N. Olaya-Galán, Ricardo Monroy, Adriana P. Corredor-Figueroa, Wilson Rubiano, Jairo de la Peña, Gertrude C. Buehring, Maria F. Gutierrez.

Methodology: Nury N. Olaya-Galán, Sandra P. Salas-Cárdenas, Milcíades Ibáñez-Pinilla, Ricardo Monroy, Adriana P. Corredor-Figueroa, Wilson Rubiano, Jairo de la Peña, HuaMin Shen.

Project administration: Sandra P. Salas-Cárdenas.

Resources: Maria F. Gutierrez.

Software: Milcíades Ibáñez-Pinilla.

Supervision: Gertrude C. Buehring, Manuel A. Patarroyo, Maria F. Gutierrez.

Validation: Jorge L. Rodriguez-Sarmiento, Milcíades Ibáñez-Pinilla, HuaMin Shen, Gertrude C. Buehring, Maria F. Gutierrez.

Visualization: Nury N. Olaya-Galán, Sandra P. Salas-Cárdenas, Jorge L. Rodriguez-Sarmiento, Gertrude C. Buehring, Manuel A. Patarroyo.

Writing – original draft: Nury N. Olaya-Galán, Maria F. Gutierrez.

Writing – review & editing: Nury N. Olaya-Galán, Jorge L. Rodriguez-Sarmiento, Milcíades Ibáñez-Pinilla, Manuel A. Patarroyo, Maria F. Gutierrez.

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6. PRESENCIA DEL VLB PROVIRAL EN FUENTES DISTINTAS A MUESTRAS DE BOVINOS: ANÁLISIS DE SU POTENCIAL ZOONÓTICO

Objetivos específicos:

3. 3. Identificar y analizar marcadores moleculares de la infección por VLB en muestras obtenidas de especies distintas al bovino y en alimentos dispuestos para consumo humano, contemplados como potenciales vías de diseminación de la infección viral.

4. Identificar factores de riesgo asociados a la adquisición del virus en mujeres colombianas.

Las enfermedades infecciosas tienen un impacto importante a nivel mundial, las cuales no solo afectan a los humanos sino también a los animales y los entornos en los cuales estos conviven (88). Antiguamente se estudiaban cada una de estas disciplinas de manera independiente, sin embargo, desde que surge el concepto de "Una Salud" (One Health) se ha integrado el estudio de las enfermedades infecciosas contemplando las dinámicas de transmisión y diseminación de los microorganismos patógenos junto con las interacciones entre especies animales y el entorno (89). De este modo se ha podido estudiar a los patógenos de una manera más eficiente, favoreciendo las estrategias de prevención y control y la implementación de políticas públicas con impactos tanto locales en cada comunidad con impacto internacional para el manejo de las enfermedades infecciosas e incluso se han planteado programas de erradicación para varios de estos patógenos (90).

Debido a procesos como la globalización, facilidad de traslado entre países, crecimiento industrial y aumento del contacto de los entornos de animales con los humanos se ha evidenciado un incremento de las enfermedades infecciosas a nivel mundial (89). El aumento de la interacción entre las interfaces compartidas entre los humanos, animales silvestres y animales domésticos, aumenta el rango de diseminación, adaptación e infección de los microorganismos hacia otros hospederos incluyendo los humanos, por tanto se ha visto registrado desde la epidemiología como un aumento en las enfermedades emergentes en los humanos (88,91). La mayoría de estas infecciones son de tipo zoonótico, descritas por la OMS como aquellas infecciones provenientes de los animales hacia los humanos, ya sea por vías directas de transmisión como el contacto con los animales y su entorno, o por vías indirectas como en el caso del consumo de alimentos o aguas

contaminadas con microorganismos de origen animal, o por el consumo de productos alimenticios provenientes de animales potencialmente infectados con microorganismos, los cuales pueden estar presentes en sus derivados como la leche, la carne o los huevos (92,93).

En el caso de los virus, el 63% de las infecciones virales que afectan a los humanos se han encontrado en otros grupos taxonómicos de animales, siendo los animales domésticos un grupo predominante en las redes de transmisión y diseminación de virus con potencial zoonótico (69). Así mismo, se ha visto en la literatura que los virus con capacidad de infección en animales domésticos tiene un rango más amplio de hospederos comparado con otros virus provenientes de otras fuentes, esto debido a las relaciones estrechas de especies domésticas que se encuentran en un mismo nicho ecológico, lo que favorece a que los virus se adapten y atraviesen las barreras interespecies (69).

Para determinar si los microorganismos encontrados en los humanos pueden considerarse como zoonóticos hay que tener en cuenta varios aspectos desde la ecología de las zoonosis: presencia y distribución del agente infeccioso en su hospedero natural, identificación de hospederos secundarios que sean susceptibles de infección, capacidad de liberación y distribución del patógeno a partir de su entorno natural, identificación de potenciales fuentes de diseminación y transmisión hacia el humano, determinación del riesgo de exposición y adquisición en los humanos, capacidad de sobrevivencia del patógeno fuera de su entorno natural y finalmente, capacidad de establecerse en los humanos permitiendo una transmisión de humano a humano (68).

Si bien no siempre todos estos aspectos se cumplen en su totalidad y, por lo tanto, no es frecuente ver que los agentes infecciosos se establezcan en el humano posterior a su transmisión inicial desde los animales y continúen su transmisión de humano a humano, en casos concretos como lo ocurrido con la actual pandemia del SARS-CoV-2, hace que las dinámicas de transmisión y la vigilancia epidemiológica sean un reto para las entidades de control y para frenar los ciclos de contagio (94). Así mismo, identificar en su totalidad las dinámicas de transmisión de estos microrganismos resulta un reto para la investigación y genera vacíos en el conocimiento, ya que hacer un seguimiento detallado de todos los factores que implican una infección zoonótica requiere de un

trabajo colaborativo interdisciplinar que toma tiempo, esfuerzo y alianzas estratégicas para llevar a cabo estudios robustos sobre dichas dinámicas.

Con respecto a la situación actual del VLB, basado en la evidencia previa de la presencia del virus en el humano registrada en el capítulo anterior de este documento, desde los inicios de la investigación del virus en el humano ha sido una incógnita cuáles son las potenciales vías de transmisión y diseminación que permiten que el virus pueda establecerse en los humanos. Como parte del planteamiento del grupo de investigación, en el cual se propone al virus como un potencial agente zoonótico, se publicó una nota al editor al inicio de la investigación, como aporte a la discusión actual en el gremio científico con respecto al VLB y su relación con el humano (Artículo 2).

Las investigaciones del VLB en su mayoría se han dedicado a entender y caracterizar la infección del virus en el ganado bovino el cual es su hospedero natural, pero poca evidencia existe en el seguimiento de las dinámicas de transmisión del virus en otros hospederos que puedan estar en contacto con los bovinos y así poder entender los ciclos completos de transmisión y diseminación que puedan estar ocurriendo, permitiendo profundizar en la ecología del virus. Por tanto, como parte de esta investigación se abordaron tres puntos clave para la determinación de infecciones zoonóticas, incluyendo la detección del virus en alimentos derivados de bovinos como la leche y la carne, considerándolos potenciales vías de transmisión hacia el humano; la detección del virus en otras especies de animales de granja como búfalos y ovejas, considerándolos como hospederos secundarios que pudiesen estar involucrados en la diseminación del virus en el entorno natural; y el análisis de segmentos génicos provirales obtenidos a partir de las fuentes previamente mencionadas, incluyendo los humanos y los bovinos con el fin de profundizar en el conocimiento de las dinámicas de diseminación y circularización del virus entre los animales y los humanos, las cuales se describen a continuación.

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Commentary Open Access

Bovine Leukemia: Zoonosis Associated with Breast Cancer in Humans?

Olaya N*, Corredor A and Gutierrez MF

Microbiology Department, Pontificia Universidad Javeriana, Colombia

***Corresponding author:** Nury Olaya, Pontificia Universidad Javeriana, Bogota, Colombia, E-mail: nuryolaya@gmail.com

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Abstract

Bovine leukemia virus (BLV) is the causative agent of enzootic bovine leukemia. It is proposed that BLV is restricted to bovines, but there are some reports of its presence in humans and its possible relationship with breast cancer. Considering that any relation or association between this virus and breast cancer could be relevant to human public health and to cattle producers worldwide, it is crucial that several research groups keep investigating in the topic with the aim of confirming the role that this virus should have in breast cancer development. Due to the lack of a vaccine, prevention and control strategies should be implemented by governments where livestock is rising.

Keywords: Breast cancer; Bovine leukemia virus; Viral tropism; Cancer association

Bovine Leukemia: Zoonosis Associated with Cancer in Humans?

Bovine leukemia is a disease reported by Leisering in 1871, associated with bovine cattle. Leisering identified in some of the animals with this disease, the development of splenomegaly and persistent lymphocytosis while most of them remain asymptomatic [1,2]. Furthermore, it was noticed that the presence of this disease could lead to important economic losses to dairy and meat herds, as well as dual-purpose bovine herds, due to the fact that milk production and weight development are affected. In addition, infected animals become susceptible to acquiring secondary infections what decreases their health conditions and thus their expected production yield [3,4].

Later on, in 1969, Miller and co-workers identified the associated pathogen to the disease, which is known nowadays as the Bovine Leukemia Virus (BLV). Afterward, it was described as an enveloped retrovirus, with a size between 80-125 nm. It is classified as a deltaretrovirus, belonging to Retroviridae family, Orthoretrovirinae subfamily. In this group, are also classified HTLVs, viruses related with neoplastic processes in humans [5]. Together with other retroviruses, their viral genome is a bicatenary RNA with positive sense polarity. It has 3 main Open Reading Frames (ORFs), which encode gag, env and pol genes with 2 Long Terminal Regions (LTRs) at the beginning and the end of the genome. In addition, it has a pX region, which has the information for auxiliary proteins for regulatory purposes such as Rex and Tax proteins, who seem to be associated with carcinogenesis process [6,7].

Until now, viral tropism has been described by the infection of B-lymphocytes as target cells in bovine cattle. However, there are some other reports that show the ability of this virus to infect different kinds of cells likewise induce damage and alterations in some other different species [8-11]. In 1984, Kettman and his group established the possibility that BLV could infect hosts like sheep, chicken, and goats, what was proven by inducing infection with in vivo assays, where, as a result, was obtained the induction of damage as it occurs in bovines [8,11]. Another important approach was performed by Levkut and co-workers who infected rabbits with the virus, and obtained clinical manifestations related to encephalitis; authors suggested that it could be understood as a zoonosis process influenced by the presence of the virus [12].

Some viruses, basically those with DNA genome as well as retroviruses, have been ascribed to the development of cancer in humans. For instance, Papillomavirus has been related to cervix cancer, Epstein-Barr virus with Burkitt lymphoma, Hepatitis B and C virus with liver cancer and HTLVs with leukemia in humans [13,14].

The relationship between virus and cancer has a long history. Some Nobel Prizes have been awarded to researchers who worked on this issue, for example: Peyton Rous was awarded in 1966 for the sarcoma virus in chickens; David Baltimore and Howard M. Temin received the prize in 1975 for their discovery of reverse transcriptase; and J. Michael Bishop and Harold E. Varmus in 1989 were honored for demonstrating that retroviral oncogenes derived from cellular genes in the host in 1976 [15].

Since then, animal and human cancers have been associated with a viral etiology in most of the cases. One of the most important examples of this association is the case of MMTV and mammary cancer development in mice, where it is established that the virus could infect directly the mammary gland, induce cellular alteration and is transmitted to progeny through the milk [16]. In further studies, another important proposal about this virus is that besides the development of breast cancer in mice, it could be also involved in the development of breast cancer and lymphomas in human beings [17,18]. Even though, there is not enough evidence of causation and a lot of controversies are still reported in the literature about this issue, what implies that future research is needed to be performed [19].

Breast cancer in humans is still one of the pathologies with highest numbers in morbidity and mortality rates worldwide [20,21], by the way, its prevalence is considerably higher in regions with frequent consumption of red meat and dairy products derived from bovine cattle [22-25]. High prevalence of breast cancer in meat-consumption countries and the fact that probably viral tropism is not exclusive to bovines, led to some researchers to seek for the presence of BLV in humans [26]; in order to know if this virus could be involved in the breast cancer development, with some hypothesis such as that it could

be infecting human cells, inducing genotypic alterations related with cellular transformation, and thus, probably having a relationship with tumor genesis in humans.

With this perspective, Dr. Buehring and co-workers' group, in the University of California, Berkeley, have performed some studies related to this topic. Their first approach was published in 2003, where they looked for antibodies in 257 human serums against the p24 viral protein, in this first study they found reactivity in 74% of the analyzed samples [26]; although the antibodies reacted against the virus, for the authors was not clear at all if the virus could be infecting humans or could be a reaction just for the viral entrance, though, authors suggested that a complete viral cycle could be occurring in humans cells due to the immunological response, but further studies are needed to clarify this concern.

Afterward, Ochoa and coworkers in Colombia [27], looked for the presence of the virus on ductal carcinoma samples, where they found that 4 out of 56 samples were positive for gp51 viral antigen in tumoral cells. In a subsequent study, this group determined the presence of a segment of the gag gene of the virus (380 pb) in human mammary tissue, with and without a cancer diagnosis [28]. As a result, authors reported the presence of the gene segment of the virus in a 40.5% of a total of 106 samples, including positive and negative cancer tissues. Particularly, authors reported that from samples with the presence of the virus, 36% belonged to positive cancer samples and 45.2% belonged to of negative cancer samples, where authors could not conclude if BLV is really associated with breast cancer development, but brings up new questions and doubts about why is there a bovine virus present in humans and if it could be influencing the development of cancer.

Last advances of Dr. Buehring's research, are trying to highly associate the presence of the virus with the development of breast cancer. In 2014, authors reported the presence of tax gene in 97 human samples for 217 samples included in the study, what raises concerns about a risk of infection of this virus to humans, and that it could be involved in the breast cancer development [29]. In her last report [30], authors established that the exposure to BLV is an important risk factor that could be highly associated with breast cancer development, with this in mind, it is important to clarify that further studies are needed with the aim of establishing a relation between the virus and the disease.

In concordance with the previous findings, the hypothesis that BLV is related to breast cancer has been formulated for a while and is getting stronger. Even though, it remains the doubt about if the virus causes cancer, is associated with cancer or just if the presence of the virus is related with the presence of the disease but without any influence in the process.

So far, although it is known that this virus causes problems in bovine public health, issues such as the difficulty of generating vaccines and the amount of asymptomatic infected animals, have been taken place to underestimate the importance of this virus by government entities and cattle keepers, until the point of having few countries with mandatory notification for this disease.

By the way, taking into account that the lack of the vaccine, as well as a specific treatment for the disease, it is necessary to include Control and Prevention strategies within farms and herds such as separating infected animals from herds, improving processes of disinfection, avoid sharing working tools or food for cattle and where possible, carry out serological diagnosis for cattle farms; together with abstaining from selling infected animals trying to control the spread of the virus and to decrease the tentative infection in humans [31].

Prevention strategies and proposals for further studies to inquire which kind of relationship this virus has with breast cancer should be executed to determine if BLV could be defined as a zoonosis associated with cancer development. Nevertheless, it is important to remind the proposals made by Joshi et al. to strongly associate causation of a virus with breast cancer [32,33]:

- The presence of viral markers: Should be evaluated in cases and controls studies, where the presence of viral markers should be greater in cases groups than in controls of the same geographic region.
- There should be a temporal relationship; virus exposure should occur before illness development.
- Association with the virus should be proven by different investigators
- Prevalence of the virus should be higher in prevalent breast cancer geographic regions
- Exposure to the virus and incidence of breast cancer should be related
- There should be a connection between transmission mode and natural course of the disease
- Oncogenic capability for the virus should be demonstrated, related with infection and transformation properties of mammary epithelial cells and causing malignancy in animal models
- Prevention of infection and spread control of the virus should decrease breast cancer incidence

Currently, our research group is working in the presence of gene segments of the virus in breast tissue samples as well as in blood, milk and meat of bovine cattle trying to elucidate any relationship between circulating strains in Colombia with the infection in humans.

As a conclusion, there has been increasing the shreds of evidence suggesting that it could be an important relationship between the presence of the virus and the breast cancer. However, even stronger findings are needed to remark this affirmation. If there would be any association, it would provide important impact in the social, economic and political field worldwide, mostly in countries with high livestock development.

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6.1. Presencia del virus en carnes y leches derivadas de bovino dispuestas para consumo humano

Con el aumento de los hallazgos en la literatura de la presencia del VLB en el humano cuyos resultados han sido confirmados a través de la secuenciación de segmentos génicos del provirus obtenidos a partir de muestras de origen humano (31,32,39), indica y confirma que este virus está presente en la población humana y que no corresponde a otro tipo de virus similar. Sin embargo, uno de los vacíos más grandes en el conocimiento está relacionado con sus vías de transmisión y de diseminación hacia los humanos. En los estudios realizados para el VLB en humanos se plantean 3 hipótesis como potenciales vías de transmisión (43,45): en primer lugar se plantea que el virus se puede estar transmitiendo a través del contacto directo con los animales, aunque esto limitaría la transmisión a personas con una actividad pecuaria directa. Las otras dos vías de transmisión hacia el humano contemplan mecanismos indirectos, como en el caso de procesos vacunales que incluyan suero fetal bovino proveniente de animales infectados (32), o por medio del consumo de alimentos derivados de bovinos infectados.

Las vías clásicas de transmisión del VLB en el ganado bovino incluyen el contacto directo con secreciones de animales infectados, durante el contacto sexual en los procesos de reproducción, a través de procesos iatrogénicos como el marcaje, descuerne, vacunación, prácticas pecuarias sin el manejo adecuado de utensilios veterinarios; y en el caso de los becerros, se ha reportado la transmisión a través de la lactancia materna (1,95,96). Si bien en el caso de los humanos no es clara la vía por la cual el virus pueda estar ingresando a este hospedero, tanto para el VLB como para otros retrovirus se reporta la transmisión a través de la lactancia materna (97–99), lo que soporta la hipótesis que pueda estar ingresando al humano por medio del consumo de alimentos derivados de bovinos infectados. Este mecanismo es una de las principales hipótesis apoyadas por diferentes grupos de investigación (31,39,43,45) que a su vez se comparte con otro tipo de infecciones zoonóticas como en el caso de la hepatitis E y brotes epidémicos asociados al consumo de carne derivada de cerdos infectados con el virus (100,101).

En Latinoamérica y Estados Unidos se registran tasas altas de consumo de productos lácteos y cárnicos de origen bovino (102–104), además, es común tener prácticas de consumo de leche cruda o de carne con baja cocción, así como el consumo de derivados lácteos con preparaciones artesanales lo que aumenta el riesgo de transmisión de infecciones provenientes de estos productos (105). Sin embargo, la falta de control y seguimiento con respecto a la calidad de los productos alimenticios que se suministran como fuente de alimento para los humanos junto con algunas prácticas culturales ponen en riesgo la seguridad alimentaria, perdiendo garantías sobre el producto final a ser suministrado para consumo (106). Una adecuada seguridad alimentaria cumple un papel fundamental para prevenir el contacto con patógenos que puedan afectar a los humanos provenientes de alimentos derivados de animales. Buenas prácticas pecuarias y hatos ganaderos sanos, garantizan una buena salud animal y así mismo previenen la diseminación de patógenos que puedan impactar tanto el entorno de los animales como de los humanos (90).

En este estudio se evaluó la presencia de segmentos génicos del provirus como indicadores de presencia del VLB en productos alimenticios derivados de bovinos como la leche y la carne, dispuestos para consumo humano (artículo 3). La carne fue obtenida de establecimientos comerciales pequeños en el área urbana de Bogotá (carnicerías) y la leche fue obtenida de granjas bovinas de producción lechera, ubicadas en la sabana de Bogotá previo al procesamiento industrial y de pasteurización. La detección de los segmentos de genoma proviral se realizó a través de una PCR anidada dirigida al gen *gag* del virus, a partir del DNA total extraído de las muestras de carnes y leches. Los productos obtenidos de las pruebas de PCR fueron confirmados por secuenciación de Sanger para verificar la identidad de los fragmentos amplificados con el VLB.

El 49% de las muestras analizadas (leches, n=24; carnes, n=25) fueron positivas para la presencia del provirus del VLB. En su momento, éste fue el primer estudio enfocado a encontrar la presencia del virus en estos alimentos de consumo, planteándolos como una potencial vía de diseminación hacia el humano. Si bien en este caso no se identificó el virus completo, estos hallazgos sugieren que los productos alimenticios provenían de animales infectados con el virus, y que potencialmente los humanos podrían estarse infectando al consumir este tipo de productos. Es claro que aún se necesitan más estudios para entender las dinámicas de transmisión de este virus hacia los humanos, pero con estos resultados se está aportando a la literatura información tendiente a demostrar esta

ingesta como punto clave de transmisión zoonótica y como un potencial mecanismo de diseminación (68).

La detección de un virus con capacidad oncogénica en productos alimenticios de consumo humano debería generarnos una alerta desde la salud pública ya que, a pesar que aún no se tiene una relación de causalidad con patologías humanas, los reportes en la literatura en diferentes grupos de investigación sugieren que este virus puede estar involucrado en procesos de cáncer, lo cual podría considerarse como un factor exógeno que se podría manejar con buenas estrategias de prevención y control, e incluso de erradicación como ya ocurre en algunos países de Europa y en Nueva Zelanda (10–12,107).

Art3: Olaya-Galán, N.N.; Corredor-Figueroa, A.P.; Guzmán-Garzón, T.C.; Ríos-Hernandez, K.S.; Salas-Cárdenas, S.P.; Patarroyo, M.A.; Gutierrez, M.F. Bovine leukaemia virus ADN in fresh milk and raw beef for human consumption. Epidemiol. Infect. 2017, 145, 3125–3130, https://doi.org/10.1017/S0950268817002229

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Bovine leukaemia virus DNA in fresh milk and raw beef for human consumption

N. N. OLAYA-GALÁN[1,2]*†, A. P. CORREDOR-FIGUEROA[2]†, T. C. GUZMÁN-GARZÓN[2], K. S. RÍOS-HERNANDEZ[2], S. P. SALAS-CÁRDENAS[2], M. A. PATARROYO[3,4] AND M. F. GUTIERREZ[2]

[1] *PhD Programme in Biomedical and Biological Sciences, Universidad del Rosario, Bogotá, Colombia*
[2] *Grupo de Enfermedades Infecciosas, Laboratorio de Virología, Departamento de Microbiología, Pontificia Universidad Javeriana, Bogotá, Colombia*
[3] *Molecular Biology and Immunology Department, Fundación Instituto de Inmunología de Colombia (FIDIC), Bogotá, Colombia*
[4] *Basic Sciences Department, School of Medicine and Health Sciences, Universidad del Rosario, Bogotá, Colombia*

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SUMMARY

Bovine leukaemia virus (BLV) is the causative agent of enzootic bovine leucosis, which has been reported worldwide. BLV has been found recently in human tissue and it could have a significant impact on human health. A possible hypothesis regarding viral entry to humans is through the consumption of infected foodstuffs. This study was aimed at detecting the presence of BLV DNA in raw beef and fresh milk for human consumption. Nested PCR directed at the BLV *gag* gene (272 bp) was used as a diagnostic test. PCR products were confirmed by Sanger sequencing. Forty-nine per cent of the samples proved positive for the presence of proviral DNA. This is the first study highlighting the presence of the BLV *gag* gene in meat products for human consumption and confirms the presence of the viral DNA in raw milk, as in previous reports. The presence of viral DNA in food products could suggest that viral particles may also be found. Further studies are needed to confirm the presence of infected viral particles, even though the present findings could represent a first approach to BLV transmission to humans through foodstuff consumption.

Key words: Bovine leukaemia virus, foodborne infection, fresh milk, raw meat, zoonosis.

INTRODUCTION

Bovine leukaemia virus (BLV) belongs to the genus *Deltaretrovirus*, family *Retroviridae*, subfamily *Oncovirinae*. This is an oncogenic virus that was first isolated in 1969 and is the aetiological agent of enzootic bovine leucosis (EBL), one of the most frequently occurring neoplastic diseases in cattle [1]; about a third of BLV-infected cows develop persistent lymphocytosis, 1–5% of them developing the late stage of the disease that is associated with B-cell neoplasm [2]. This retrovirus is closely related to the types of human lymphotropic T-cell leukaemia virus (HTLV-1 and -2) [3]. BLV integrates its genome in target bovine cells, so that all infected animals are persistently infected and become carriers of the virus during the course of

* Author for correspondence: N. N. Olaya-Galán, Grupo de Enfermedades Infecciosas, Laboratorio de Virología, Departamento de Microbiología, Pontificia Universidad Javeriana, Carrera 7 No. 40 – 62, Building 50 Lab. 123, Bogotá, Colombia.
(E-mail: nuryolaya@gmail.com)
† These authors contributed equally to this work.

their lives, thereby having a negative impact on the animals' immune system and, consequently, induces losses in milk production, poorer yield regarding weight and induced abortions in the animals [1, 4, 5].

A few studies have revealed the presence of DNA and proteins of BLV in human breast tissue samples, proposing that these findings could be considered as a hazardous factor for breast cancer development [6–9]; albeit some different investigations have not found evidence of the virus in people [10, 11], there is proof that a bovine virus is found in human beings, perhaps related to a zoonosis infection. However, it is still not known how BLV is transmitted to humans. Transmission in cattle could be mediated by horizontal or vertical transmission. Vertical transmission includes perinatal transmission through blood or transplacental passage and post-natal infection routes through colostrum and milk consumption [12, 13]. Horizontal transmission could occur by direct contact between infected and non-infected animals, as well as veterinary practices due to using contaminated instruments on many animals without sterilising them between procedures and animals, including tattooing cattle, vaccination and rectal palpations [14]. Conversely, it has been suggested that humans might become infected by consuming foodstuffs from infected animals, through the direct contact involved in livestock practices or vaccines produced with contaminated cattle sera [7, 15]. Although the transmission route has not yet been established, it could be implicated in as-yet-unknown human health issues such as the emergence of new diseases, taking into account that BLV is described as an oncogenic virus.

Food for human consumption has been proposed as a potential source of pathogen transmission. Several viral infections are related to foodborne diseases; enteric viruses, such as rotaviruses and noroviruses, are amongst the viral agents most frequently transmitted by foodstuffs; these agents are transmitted as free viral particles through faecal contamination of foodstuffs for direct consumption, such as fruit and vegetables and (in some exceptions) meat products [16, 17]. Some other viral infections require the presence of infected cells to transmit the agent to other hosts; this is the case of hepatitis A virus and hepatitis E virus (HEV) that have been found in meat products, such as sausages, liver and pork [18–21].

There are other diseases that are, in principle, associated with foodstuff consumption but where the causative agent remains unknown [22]; in spite of most of them being associated with gastrointestinal diseases, there could be other types of pathogens in foodstuffs that are still unknown. This could thus be happening with BLV, involving potential risk for human health. Studies focused on food safety for improving the quality of products prepared for human consumption are needed as foodstuffs could transmit unknown pathogens. This study was thus aimed at evaluating the BLV DNA detection in raw meat and fresh milk (i.e. fresh from milking) for human consumption as a first step in estimating the potential of foodstuffs regarding BLV transmission to humans.

METHODS

Sample collection

Convenience sampling was used for obtaining both milk and meat samples, 100 samples were obtained. Fifty beef samples weighing around 15 g each were obtained from butchers in Bogotá whilst the 50 samples of milk were obtained from farms specialising in dairy production located in different parts of Colombia. The milk was collected directly from milking (i.e. before being sent for industrial treatment). The samples were transported to the Virology Laboratory at the Javeriana University in Bogotá where meat samples were stored at −20 °C until being processed, whilst milk samples were processed immediately.

Sample preparation and nucleic acid extraction

Roche High Pure PCR Template Preparation Kit was used for extracting total nucleic acids from milk and meat, following the manufacturer's indications; some modifications were made for solid tissue and liquid samples. Regarding meat, an initial 10–20 mg of rump cut (muscle) was lysed with proteinase K and the tissue lysis buffer supplied in the DNA extraction kit. Extraction from milk samples first involved cell concentration from an initial 5 ml milk volume through sequential centrifugations at 16 000 ***g*** for 20 min for each cycle (four cycles in total); the pellet so obtained was used for DNA extraction, following the manufacturer's instructions. NanoDrop (Thermo) was used for quantifying the extracted DNA to verify its concentration and purity. The DNA was then frozen (−20 °C) and stored until further use.

PCR amplification: multiplex and nested PCR

The bovine GAPDH constitutive gene was used as PCR internal control, which was amplified in a

multiplex PCR together with the virus' *gag* gene encoding its capsid proteins. PCR tests were done using Roche PCR master mix with specific primers (0·8 pmol/µl) for the aforementioned genes. Both bovine GAPDH and *gag* primers were previously reported by Buehring *et al*. [6]. An 857 bp fragment was amplified for the bovine GAPDH gene and a 385 bp fragment for *gag*. Multiplex PCR conditions included an initial denaturing step at 94 °C for 5 min, followed by 35 cycles of denaturing at 94 °C for 30 s, an annealing step at 59·3 °C for 60 s and a 90 s extension at 72 °C. A final extension step was performed at 72 °C for 10 min.

PCR sensitivity was increased by nested PCR for samples where the viral gene was not amplified in multiplex PCR. The first PCR's products were used as templates for the nested PCR. The amplification target was an internal *gag* fragment (nt 1097–1369), resulting in a 272 bp fragment (also reported by Buehring *et al*.) [6]. Reaction conditions were the same as those described for multiplex PCR. Annealing temperature was 56 °C with 30 s extension time.

The results for both multiplex and nested PCR were visualised on 1·5% agarose gels prepared in 1× TAE (Biorad) dyed with 1× HydraGreen fluorescent intercalating dye (ACTGene). DNA extracted from a blood sample of an infected animal was used as positive control for BLV and RNase- and DNase-free water as negative amplification control.

Sequencing

Virus-positive PCR products were purified with PCR product purification kit (Roche High Pure), following the manufacturer's instructions, and then sent to Macrogen Inc. (Seoul, Korea), for Sanger sequencing. The primers (both sense and anti-sense) used for sequencing were the same as those for the nested PCR. BioEdit Sequence Alignment Editor (version 7·2·5) was used for editing and analysing the sequences. Consensus sequences were obtained for each positive sample; the online BLASTn tool was used for verifying the identity of the sequences so obtained.

RESULTS AND DISCUSSION

BLV has been known and studied as an infectious agent in cattle; however, there are reports of this virus in humans [6, 7, 9] even though the mechanism by which the virus has reached such host has still not been clarified. Buehring *et al*. [15, 23] highlighted three main hypotheses for the viral entry to humans. The first considers direct contact with infected animals; however, such hypothesis would necessarily involve viral presence in limited populations (i.e. veterinarians, livestock handlers and/or farmers). Nevertheless, available evidence has revealed that the virus has been found in people who do not necessarily come into direct contact with animals [6, 7, 9]. The second hypothesis concerns possible BLV transmission through vaccine production processes involving the use of BLV-contaminated foetal bovine sera, even though no experimental evidence has been published regarding this issue. The third hypothesis proposes that the virus might infect humans through the consumption of bovine-derived products from BLV-infected cattle [10, 23], leading to the idea of evaluating meat and milk products for human consumption as a possible pathway for viral entry.

Nucleic acids were obtained from fresh milk and raw beef samples (muscle tissues) in the present study in the search for BLV proviral DNA. Internal control (bovine GAPDH) was observed in the multiplex PCR (Fig. 1*a*). A proviral *gag* segment was found in 24 out of the 50 milk samples and in 25 of the meat samples. These results represent 49% of all samples analysed. Most of them were detected by nested PCR, suggesting that the viral load in the samples was considerably low. Figure one shows a representative agarose gel of the results obtained by multiplex PCR (a) where an 857 bp fragment from bovine GAPDH was observed, as well as the external *gag* fragment in positive control (385 bp); nested PCR (b) from the products obtained in the first PCR with a 272 bp fragment was observed in positive control and the samples analysed here (Figs. 1*a* and *b*).

After sequencing PCR products, *gag* gene identity was verified using BLAST (NCBI) with previously reported BLV sequences. The results gave 97–99% identity compared to reference sequences. Such results confirmed that the amplified products obtained from meat and milk samples came from the BLV *gag* segment.

BLV prevalence in Colombia has been recorded as 67·7% on livestock farms throughout the country and in 43% of the bovine population, thereby affecting (health-wise and economically) livestock breeding for milk production and meat for human consumption [24]. Understanding the evolution of the disease in cattle (most infected animals going unnoticed due to low symptomatology) [25] and considering its high prevalence in Colombia highlights the fact that infected

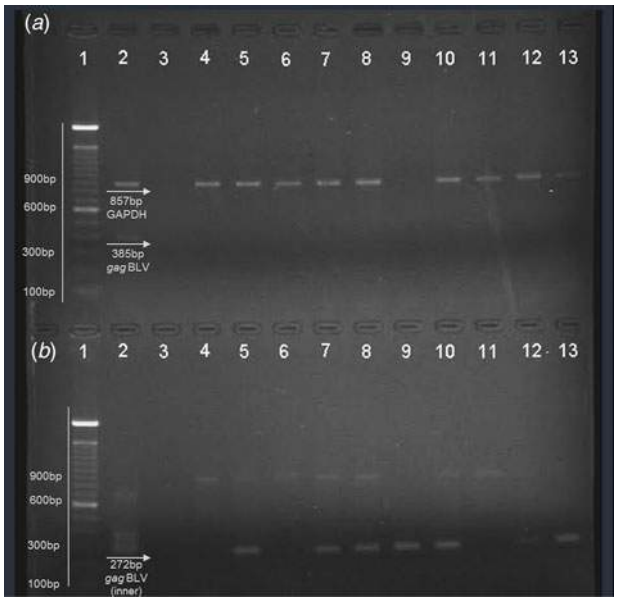

Fig. 1. Representative agarose gel (1·5%) of field samples. (*a*) Multiplex PCR. (*b*) Nested PCR. Lane 1 – 100 bp ladder (Invitrogen); lane 2 – positive control; lane 3 – negative control; lane 4–8: beef samples; lane 9–13: milk samples.

animals that have never been detected could be sold for commercialisation, and thus cattle-derived infected products could be distributed in different industries without any government control regarding the presence of the virus. This would favour the disease's dissemination in spite of the fact that EBL was recently established and was considered a disease requiring mandatory notification by the Colombian Agricultural and Livestock Institute [26].

Detection of BLV DNA in cattle-derived foodstuffs (as shown by this study's findings) could serve as a marker, which could suggest a zoonosis (i.e. indicating viral particle transmission by these products). It is worth stressing that cases of zoonosis are considered one of the most important problems regarding infectious disease epidemiology and public health worldwide [27]. Taking the WHO's definition of zoonosis, 'Any disease or infection that is naturally transmissible from vertebrate animals to humans, including all types of pathogenic agents', [28] as well as viral transmission mechanisms, action directed towards avoiding viral dissemination in cattle might prevent the introduction of the pathogen into the human population, even though BLV has not yet been conclusively proven to be a cause of human disease [6, 11, 29].

Foodborne diseases are related with ingesting contaminated foodstuffs with microorganisms, which sometimes could come from an animal origin [30]. Two vehicles have been proposed for viral transmission through foodstuff consumption. Free viral particles in foodstuffs has been related to an exogenous contamination source (i.e. faecal contamination), involving direct consumption of fresh products, such as fruit and vegetables [16]. The other possibility concerns the transmission of viruses through animal-derived products infected with the virus. In this case, animal cells would become carriers of pathogenous agents, introducing them into human beings through consumption of meat from infected animals, trespassing even free viral particles, infected cells or proviral DNA [31]. Reports regarding HEV entry to humans have increased due to products from pigs infected by the virus being consumed, i.e. sausages, liver and poorly cooked pork [18, 19, 21, 32]. This situation has led to acute hepatitis outbreaks where a hitherto disregarded zoonosis has been clearly defined. This study's findings have suggested that a similar situation could be occurring with BLV, giving rise to possible explanations for diseases whose causes have previously been undefined.

The pertinent literature has reported viral particles and viral DNA in cows' milk or colostrum that could be considered a risk factor for transmission to calves [33–36]. Viral DNA was also detected in fresh milk in the present study, thereby agreeing with previous reports, despite not having been described as a risk factor for humans. Bearing this in mind, some other retroviruses could be transmitted by their respective hosts consuming milk, i.e. HTLV, MMTV (mammary murine tumor virus) and also HIV in particular situations [37–39].

Consuming raw milk could be a viable transmission pathway, mostly in developing countries having high raw milk consumption in rural populations. Previous studies have established that industrialisation of milk and pasteurisation processes leads to inactivating viral particles [40–42]; avoiding raw milk consumption would thus be an essential prevention strategy, even if it remains unknown whether BLV can also reach humans by this means.

It is worth highlighting that even though the study's objective was not to determine the presence of complete and infectious viral particles in the samples analysed, the gene fragments found here suggested the virus could be found, since comparing the sequences obtained here with previously reported ones gave 97–99% identity (i.e. dealing with BLV). Further studies should be aimed at establishing whether consuming the aforementioned foodstuffs transmits infective viral particles, which can then complete their biological cycle in humans. It could be of great interest to

evaluate viral presence after cooking meat as this could inactivate viral particles and to ascertain whether other mechanisms could be participating in viral transmission [40].

Moreover, this is the first experimental approach reporting the BLV *gag* gene segment being detected in beef products for human consumption. Questions concerning viral transmission through consuming infected meat have been raised since the reports by Buehring *et al*. [15]. However, only empirical approaches and inferences about this transmission pathway have been proposed, regarding slaughterhouse practices involving carcinogenic cattle tissue where not only these tissues are distributed to humans, but have been disposed of for dog and cat food products [43]. The data reported here are important for foodborne infections and public health. Prevention policy, which proposes the early detection of pathogenous agents with a possibility to reach humans, depends on the risks for the target population, by controlling the main sources of dissemination [44].

The presence of the BLV DNA in bovine-derived products could be interpreted as a step forward in identifying previously unknown foodborne diseases. Our results suggested that BLV could be considered a potential zoonotic agent, even though non-infectious particles were reported in this study. Evidence of an oncogenic virus' DNA in milk and meat products highlights such foodstuffs as being a potential source of viral transmission to humans and could be the outcome of currently unknown diseases. Such viruses' alternative transmission routes should be studied (i.e. human-to-human transmission). Prevention and control strategies should be enforced to decrease viral prevalence and transmission in cattle and ensure that infected foodstuffs do not become distributed to markets; such alternatives aimed at eradicating the disease have been achieved in some European countries, New Zealand and Australia [5, 45].

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DECLARATION OF INTEREST

None.

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6.2. Presencia del virus en especies animales distintas a bovinos: infección natural de ovejas y búfalos

Otro de los factores importantes de las infecciones zoonóticas se asocia con la presencia de hospederos intermediarios o accidentales que puedan participar en procesos de adaptación, transmisión y diseminación de agentes patógenos favoreciendo el intercambio de estos entre especies, aumentando el rango de hospederos susceptibles de infección y facilitando las rutas de diseminación hacia los humanos (69). Esto representa un reto importante para las entidades de control tanto de la salud humana como de salud animal ya que en el caso de presentarse múltiples hospederos participando en las redes de transmisión, se hace más difícil identificar y manejar las fuentes primarias de diseminación de patógenos (90,108).

Como parte de las estrategias de control bajo la perspectiva de "One Health", la vigilancia epidemiológica, el seguimiento de las infecciones y el diagnóstico de los patógenos en sus entornos naturales son fundamentales para poder tener un manejo integrado y un mejor conocimiento de las dinámicas de transmisión y diseminación de los mismos (90). Asimismo, bajo este principio deberían considerarse otras especies animales diferentes a los hospederos naturales que puedan ser susceptibles de infección, sobre todo aquellos que se encuentren en nichos ecológicos compartidos entre especies favoreciendo los ciclos de transmisión de los patógenos.

América Latina es una región en donde el crecimiento de la industria pecuaria ha aumentado de manera significativa en las últimas décadas convirtiéndose en una región importante para los procesos de exportación (109). En Colombia y en otras regiones de Latinoamérica es frecuente encontrar explotaciones ganaderas mixtas en las cuales se comparten espacios en las fincas con diferentes tipos de animales como el ganado vacuno, ovino, bufalino, caprino e incluso porcino y de aves de corral (110). Este tipo de granjas pecuarias aumentan el riesgo de transmisión de enfermedades infecciosas entre especies, lo que dificulta el seguimiento y control de la propagación de patógenos en los animales (104). Si bien desde la Organización Mundial de Sanidad Animal (OIE) existen listas de microorganismos que deberían diagnosticarse en las diferentes explotaciones pecuarias (111), la persistente carencia de recursos para implementar

pruebas diagnósticas de rutina en la región de América Latina es un factor crítico para el seguimiento epidemiológico de las dinámicas de transmisión de estos patógenos (104,105).

El aumento de los brotes de enfermedades infecciosas entre los animales implica pérdidas económicas y afectaciones en la salud animal ya que aumenta el uso de medicamentos, el rendimiento en producción disminuye y la calidad de los productos derivados de los animales se ve afectado en términos de seguridad alimentaria (106). Estos factores se convierten en una preocupación en el gremio ganadero ya que generan mayores esfuerzos en términos de gestión y logística para garantizar las buenas prácticas pecuarias y para no afectar el producto final, ya que esto podría reducir la comercialización y distribución de los productos de origen animal (104).

Para el caso del VLB, se ha propuesto como un agente versátil que tiene capacidad de infección de múltiples especies ya que en estudios previos se ha identificado la presencia del virus por infección natural en otras especies animales diferentes al ganado vacuno como las ovejas, cabras, yaks, búfalos y alpacas (16,18,112,113), los cuales podrían actuar como hospederos intermedios que participen en los ciclos de transmisión del virus, e incluso que aumenten el riesgo de la diseminación del virus hacia los humanos al tener un mayor rango de especies susceptibles de infección.

Por tanto, desde la visión de un trabajo colaborativo integrando disciplinas como la veterinaria y la investigación y con aras de aportar a la vigilancia epidemiológica del VLB en Colombia, se planteó un estudio para la detección del provirus en otras especies de interés en el sector pecuario del país, como los búfalos y las ovejas, que son especies que han venido en crecimiento en el sector pecuario como fuente de alimento para consumo humano y que además es común encontrar explotaciones mixtas de estas especies en las diferentes regiones ganaderas del país (114) (Artículo 4). Debido a que no había antecedentes de la presencia del virus en estas especies en Colombia se realizó un estudio descriptivo de tamizaje poblacional para determinar la presencia del virus en ovejas (n=44) y búfalos (n=61). Se tomaron muestras de sangre de los animales provenientes de diferentes regiones del país en las cuales es común las explotaciones mixtas. Se evidenció la presencia del provirus mediante la detección de un segmento del gen *tax* por PCR anidada. Se encontró la presencia del provirus en el 34% de las muestras de ovejas (n= 15) y 19.6% de los

búfalos evaluados (n=12). Para confirmar los resultados se secuenciaron 10 de los productos de PCR amplificados por tecnología de Sanger y se encontró una identidad mayor al 99% al compararlas con secuencias previamente reportadas de genoma completo del VLB en el GenBank. Se realizó un análisis filogenético de máxima verosimilitud con el set de datos y se realizó un análisis complementario del receptor celular de las ovejas y los búfalos, así como su interacción con la proteína gp51 del virus, la cual tiene capacidad de unión al receptor celular AP3D1 del bovino.

Estos resultados demostraron la presencia del provirus en otras especies animales de importancia en el sector pecuario en Colombia, lo que sugiere que el virus está cruzando la barrera de especies y que otros animales pueden estar involucrados en sus ciclos de transmisión e incluso se puede estar aumentando el riesgo de diseminación hacia el humano, al aumentar el rango de hospederos a los cuales el humano pueda tener acceso directo o indirecto debido a las prácticas pecuarias o al acceso de productos derivados de los animales. Estos resultados demuestran la importancia de plantear estrategias integradas de prevención y control en el sector pecuario para trabajar en conjunto buscando una mejor salud animal y mejor garantía en la seguridad alimentaria para los productos alimenticios provenientes de los animales de granja, ya que tanto los búfalos como las ovejas son comercializados como fuentes de carne y de leche dispuestas para consumo humano (115,116).

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ORIGINAL ARTICLE

Evidence of bovine leukemia virus circulating in sheep and buffaloes in Colombia: insights into multispecies infection

Nury N. Olaya-Galán[1,2] · Adriana P. Corredor-Figueroa[3] · Sebastián Velandia-Álvarez[1] · Diana S. Vargas-Bermudez[4] · Nathalia Fonseca-Ahumada[1] · Kerlimber Nuñez[1] · Jairo Jaime[4] · María Fernanda Gutiérrez[1]

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Abstract

Bovine leukemia virus (BLV) is the causative agent of leukemia/lymphoma in cattle. However, previous evidence has shown its presence in other species of livestock as well as in humans, suggesting that other species can be accidental hosts of the virus. In viral infections, receptors that are common to different animal species are proposed to be involved in cross-species infections. For BLV, AP3D1 has been proposed to be its receptor, and this protein is conserved in most mammalian species. In Colombia, BLV has been reported in cattle with high prevalence rates, but there has been no evidence of BLV infections in other animal species. In this study, we tested for the virus in sheep (n = 44) and buffaloes (n = 61) from different regions of Colombia by nested PCR, using peripheral blood samples collected from the animals. BLV was found in 25.7% of the animals tested (12 buffaloes and 15 sheep), and the results were confirmed by Sanger sequencing. In addition, to gain more information about the capacity of the virus to infect these species, the predicted interactions of AP3D1 of sheep and buffaloes with the BLV-gp51 protein were analyzed *in silico*. Conserved amino acids in the binding domains of the proteins were identified. The detection of BLV in sheep and buffaloes suggests circulation of the virus in multiple species, which could be involved in dissemination of the virus in mixed livestock production settings. Due to the presence of the virus in multiple species and the high prevalence rates observed, integrated prevention and control strategies in the livestock industry should be considered to decrease the spread of BLV.

Introduction

The livestock industry is one of the most important areas of economic activity in Latin America and is a major source of food and income for the communities [1]. Due to its location,

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Nury N. Olaya-Galán and Adriana P. Corredor-Figueroa have contributed equally as first authors.

✉ María Fernanda Gutiérrez
mfgutier@javeriana.edu.co

Nury N. Olaya-Galán
nury.olaya@urosario.edu.co

Adriana P. Corredor-Figueroa
acorredorf@ecci.edu.co

Sebastián Velandia-Álvarez
velandia.s@javeriana.edu.co

Diana S. Vargas-Bermudez
dsvargasb@unal.edu.co

Nathalia Fonseca-Ahumada
luisafonseca@javeriana.edu.co

Kerlimber Nuñez
nunez_k@javeriana.edu.co

Jairo Jaime
jjaimec@unal.edu.co

1 Grupo de Enfermedades Infecciosas, Laboratorio de Virología, Departamento de Microbiología, Pontificia Universidad Javeriana, Bogotá, Colombia

2 PhD Program in Biomedical and Biological Sciences, Universidad del Rosario, Bogotá, Colombia

3 Universidad ECCI, Calle. 51 # 19–36, Bogotá DC, Colombia

4 Departamento de Salud Animal, Centro de Investigación en Infectología E Inmunología Veterinaria (CI3V), Facultad de Medicina Veterinaria Y de Zootecnia, Universidad Nacional de Colombia, Sede Bogotá, Carrera 30 No. 45-03, 1100 Bogotá, Colombia

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proximity to the equator, climate, and broad landscapes, Latin America has become in one of the largest producers of livestock worldwide [1, 2]. Mixed crop/livestock systems are common in Latin America, with multiple animal species raised together on the same farms [3, 4]. Although some studies have demonstrated the advantages of multi-species farming systems [5–8], these systems require increased labor and management on the farms. As the demand for livestock increases, new challenges also should be considered, such as the management of mixed-species animal herds in terms of food supplies for the animals, water sources, spatial distribution, and control of infectious disease outbreaks [9]. Higher risks of infectious disease transmission might exist due to interactions between different species, favoring the spread and expansion of the host range of microorganisms among animal species and even humans, leading to emerging infectious diseases [8, 10].

One of the most important concerns about these infections in multiple animal species is that some animals could remain asymptomatic, serving as intermediate hosts and playing a crucial role in the dissemination of pathogens. Outbreaks of infectious diseases among animals result in economic losses due to the need for treatment, reduction in productivity, early culling of infected animals, and restrictions to their commercial use [10]. Recent outbreaks in mixed livestock production facilities have been reported for both viruses and bacteria, indicating the occurrence of interspecies infections, coinfections with multiple pathogens, and dissemination of multi-drug-resistant bacteria [11, 12]. Crossing the species barrier also increases the risk of spillover to humans [13], as pathogens develop new features, with high mutation rates and recombination processes leading to the emergence of infections with zoonotic potential [8, 10].

Bovine leukemia virus (BLV) is a retrovirus with oncogenic potential and is the etiological agent of enzootic bovine leukosis, which is distributed worldwide with high prevalence rates, particularly in Latin America [14]. BLV establishes a persistent infection for the entire life of the animal, but more than 70% of infected animals remain asymptomatic. The other 30% develop persistent lymphocytosis, and between 5 and 10% develop leukemia/lymphoma, which is the most advanced stage of the disease [15]. BLV infection leads to economic losses in the livestock industry, as it increases the risk of secondary infections in the host, decreases milk production, favors weight loss, and increases the risk of abortions and other unfavorable clinical outcomes [16, 17].

BLV appears to be a versatile infectious agent, as evidence of natural infections has been reported in multiple animal species [18, 19], including buffaloes [20], yaks [21, 22], sheep [23, 24], and alpacas [25]. In addition, BLV has been found in breast tissue [26, 27], lungs [28], and blood [29] of humans, suggesting the occurrence of zoonotic infections, which might be associated with cancer development [30–32]. Recently, co-circulation of the virus among humans and cattle was also reported, with evidence of transfer of molecular signatures and genetic flux between species [33]. This suggests that food products obtained from infected cattle could be a potential source of viral dissemination and zoonosis [34]. One of the proposed mechanisms of cross-species infection is the use of receptors that are conserved among species, allowing viruses to replicate in both natural and accidental hosts [35]. For BLV, transport proteins such as AP3D1 (adaptor-related protein complex-3) [36, 37] and CAT1/SLC7A1 (cationic amino acid transporter-1/solute carrier family 7 member 1) [38] have been identified as potential receptors in cattle. These proteins interact with the gp51 Env protein, which is located in the viral envelope and mediates viral entry into the host cells [39]. In our research group, interactions between bovine AP3D1 and the gp51 Env protein of BLV have been characterized using both *in silico* and *in vitro* techniques [36]; however, there is no evidence of interactions with the corresponding proteins of other animal species, despite the presence of the virus in other animal hosts.

In Colombia, BLV was detected recently with high prevalence rates (62%) in the cattle industry, distributed throughout the main livestock production regions in the country [40]. The livestock industry in Colombia, and in Latin America in general, has a strong impact on its economic development, with a significant expansion in the last decade [2]. Mixed livestock farms are common in Colombia, particularly those with cattle, sheep, and buffaloes, which are species that have undergone a significant increase in their populations. Both sheep and buffaloes are raised commercially as sources of meat for human consumption and for production of milk and dairy products [41, 42]. Although Colombia has an epidemiological surveillance system for animal health administered by ICA (Instituto Colombiano Agropecuario), there are still gaps in the diagnosis and control of infectious diseases listed in the OIE Terrestrial Animals Health Code [43]. Enzootic bovine leukosis is considered a disease of concern by the OIE [44], but unfortunately, is not included in the official standards of control of animal diseases in Colombia, and thus, diagnosis is not supported by the government [45, 46]. Considering the high prevalence of BLV in Colombian cattle [40] and evidence of the virus in other livestock species in other regions, this study was aimed at determining whether the virus is circulating in sheep and buffaloes in Colombia, as no studies are available that focused on these species. Likewise, as part of the supporting evidence of the natural infection of these species, *in silico* modeling of the AP3D1 proteins of buffaloes and sheep and their interactions with the gp51 protein of BLV was performed.

Materials and methods

Analysis of AP3D1 of multiple species and interactions with gp51 of BLV

Retrieval and multiple alignment of AP3D1 sequences

To investigate the plausibility of natural transmission of BLV to other species present on mixed farms, as well as its zoonotic potential, sheep, goat, buffalo, deer, alpaca, pig, horse, and human AP3D1 sequences were retrieved from the National Center for Biotechnology Information (NCBI, USA) database and compared to the bovine AP3D1 sequence as a reference (Table 1). Multiple sequence alignment (MSA) was performed using the ClustalW tool available in MEGA7 software [47]. The final alignment was visualized and edited using Jalview Ver2.11.1.4 [48].

AP3D1 and Env protein 3D structures and docking simulations

Protein structures used in this study were predicted previously by our research group [36]. Briefly, COPI (PDB ID 5A1U) and AP2 adaptor complex (PDB ID 2VGL) crystal structures were used as templates for modeling the 3D structures of Env and bovine AP3D1, respectively. To avoid the need to build a new model for the AP3D1 protein of each species, the mutation wizard function in PyMOL [49] was used to mutate the amino acids that were different in the BLV receptor (BLVR) domain of AP3D1 of buffalo and sheep based on the MSA described above, using the bovine AP3D1 model as a template.

Each of the AP3D1 structural models was then used for molecular docking to the gp51 region of Env protein of BLV, using HADDOCK software [50]. For the docking protocol, residues in Env gp51 and boAP3D1 that were identified previously as being relevant for binding between the two proteins were included in the model: 97A, 98S, 115H, 127W, 128E, and 170N for gp51 and 695D, 800R, 807D, and 925K for AP3D1 [36]. Residues surrounding the active region were selected as passive in the model, and all of them could interact indirectly. HADDOCK scores for the interactions were also obtained.

Table 1 Accession numbers for AP-3 complex subunit delta-1 (AP3D1) protein sequences of cattle, humans, and ungulate species

Accession number	Species
NP_003929.4	*Homo sapiens*
NP_776423.3	*Bos taurus*
XP_005682664.1	*Capra hircus*
XP_006206482.1	*Vicugna pacos*
XP_020761624.1	*Odocoileus virginianus*
XP_020939857.1	*Sus scrofa*
XP_023500114.1	*Equus caballus*
XP_024849589.1	*Bos taurus*
XP_025148895.1	*Bubalus bubalis*
XP_027402912.1	*Bos indicus*
XP_027825737.1	*Ovis aries*

Screening of BLV circulating in sheep and buffaloes in Colombia

Population and sample collection

As no previous evidence of BLV in buffaloes and sheep in Colombia was available, a convenience sampling strategy was used for virus detection in these species. Figure 1 shows the regions where mixed production facilities are present. Currently, there are about 28 million bovines, 1.7 million sheep, and 500,000 buffaloes in these regions [51], with a BLV prevalence of 62% in cattle [40]. Blood samples were taken from 61 buffaloes and 44 sheep from different departments of the country. Buffalo samples were collected on farms in Antioquia (n = 40) and Cundinamarca (n = 21), and sheep samples were obtained from slaughterhouses located in Cundinamarca (n = 22) and Santander (n = 22) (Fig. 1). On average, the buffaloes were about 2-3 years of age, and the sheep were 5-6 months old.

Whole blood samples were collected in Vacutainer tubes with EDTA and were shipped to the virology lab at the Pontificia Universidad Javeriana (Bogota) for processing. Mononuclear cells were separated by density gradient centrifugation using LymphoSep separation medium (MP) following the manufacturer's instructions. Total DNA was extracted using a High Pure PCR Template Preparation Kit (Roche) as instructed by the manufacturer. Total DNA was stored at -20°C until later use.

Detection of BLV proviral DNA and phylogenetic analysis

The integrity and quality of the extracted DNA was verified by the amplification of constitutive genes for both species by conventional PCR. bovGAPDH (856 bp) and cytochrome C (267 bp) housekeeping genes were used for buffaloes and sheep, respectively. Samples that were negative for housekeeping gene amplification were excluded from the study.

BLV detection was carried out by nested PCR targeting a region of the *Tax* gene of the virus (284 bp). Primers and PCR conditions used in this study were reported previously [26]. Tests were carried out using PCR master mix (Sigma-Aldrich) following the instructions of the manufacturer, with a final volume of 25 µL and a primer

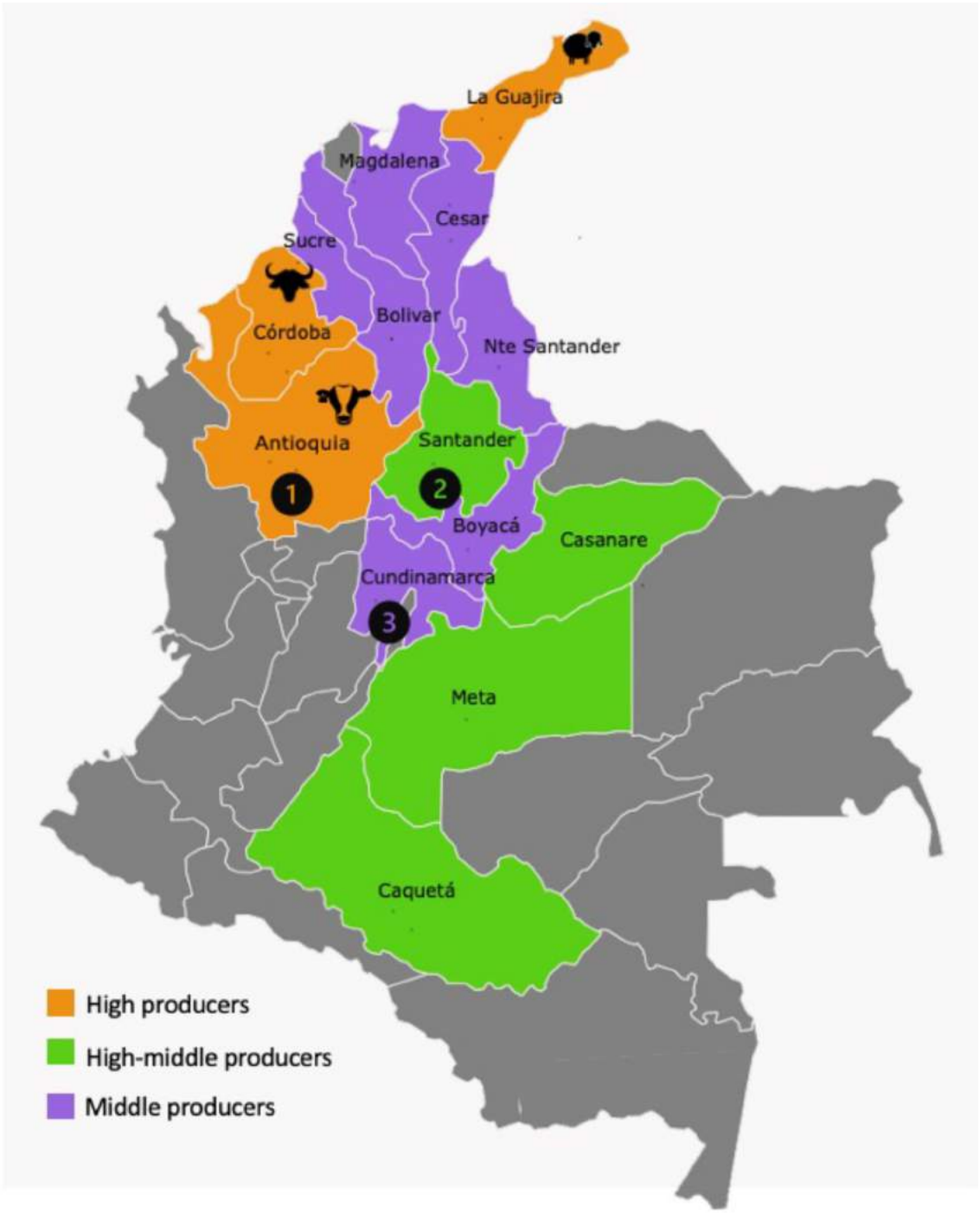

Fig. 1 Regions with mixed-species livestock production in Colombia. Antioquia, Cordoba, and La Guajira are the departments with the highest production of cattle (n = 3,000,000), buffaloes (n = 90,000), and sheep (n = 800,000). Colors indicate the population density of animals of multiple species, divided into high producers (>2 million animals), high-middle producers (1–2 million animals), and middle producers (300,000– 1 million animals) [51]. *Regions in gray represent small or non-producers with fewer than 300,000 animals in the department. Numbers indicate the locations where samples were collected. Buffalo samples were collected in Antioquia (1) and Cundinamarca (3), and sheep samples were collected in Santander (2) and Cundinamarca (3). The map was created with Pixel Map Generator, available online (https://pixelmap.amcharts.com)

concentration of 0.8 pmol/μL. Amplicons were analyzed by electrophoresis in 1.5% agarose gels in TAE buffer, stained with Diamond Nucleic Acid Dye (Promega). DNA obtained from a fetal lamb kidney (FLK) cell line persistently infected with BLV was used as a positive control. DNase/RNase-free, molecular-grade water was used as a negative control.

PCR products from buffalo and sheep samples with the highest DNA concentrations were purified and shipped to the SIGMOL sequencing service of Universidad Nacional, Colombia, for Sanger sequencing. An inner 115-bp region of the amplified PCR product was used for sequencing, using the primer set F (TGTCACCATCGATGCCTG G)/R (CATCGGCGGTCCAGTTGATA). The identity of the sequences was verified using BLASTn. Sequences obtained from buffaloes (n = 4) and sheep (n = 3) were compared with 97 complete BLV genome sequences retrieved from the GenBank database. Information about the sequences and FASTA files are available in the supplementary materials of this article. Sequences were aligned by the ClustalW method in MEGA7 [47]. The best evolutionary model was selected automatically using the "Find Model" option integrated in MEGA software, which is based on the Akaike information criterion (AIC) [52]. Phylogenetic reconstruction was also performed in MEGA7 by the maximum-likelihood method, with 1000 bootstrap pseudoreplicates. FigTree program Ver1.4.4 was used to visualize and edit the phylogenetic tree (http://tree.bio.ed.ac.uk/software/figtree/).

Results

AP3D1 sequence alignment and molecular docking simulations

AP3D1 sequences retrieved from the GenBank database were filtered for the proposed BLVR domain in cattle (aa 660-807) [36]. A multiple alignment was performed to identify regions that are conserved among species. Figure 2 shows an alignment of AP3D1 amino acid sequences using boAP3D1 as a reference. The residues predicted to interact with BLV-gp51 are colored in red, and these were found to be conserved in all of the species compared. Sequence variations were found only in regions that were not predicted to interact with BLV-gp51. All of the sequences were at least 95% identical to that of bovine AP3D1.

Since the most common livestock species raised together on farms in Colombia are cattle, sheep, and buffaloes, the AP3D1 proteins of these species were selected for modeling of molecular docking. The BLVR domain of buffalo AP3D1 differed from that of cattle by only two amino acid substitutions, R734Q and V777I, whereas in sheep, the BLVR domain was identical to that of cattle.

Figure 3 shows the results of docking simulations using sheep or buffalo AP3D1 and gp51. In both models, the region interacting with gp51 belongs to the BLVR domain. The HADDOCK score and binding affinity of the complex

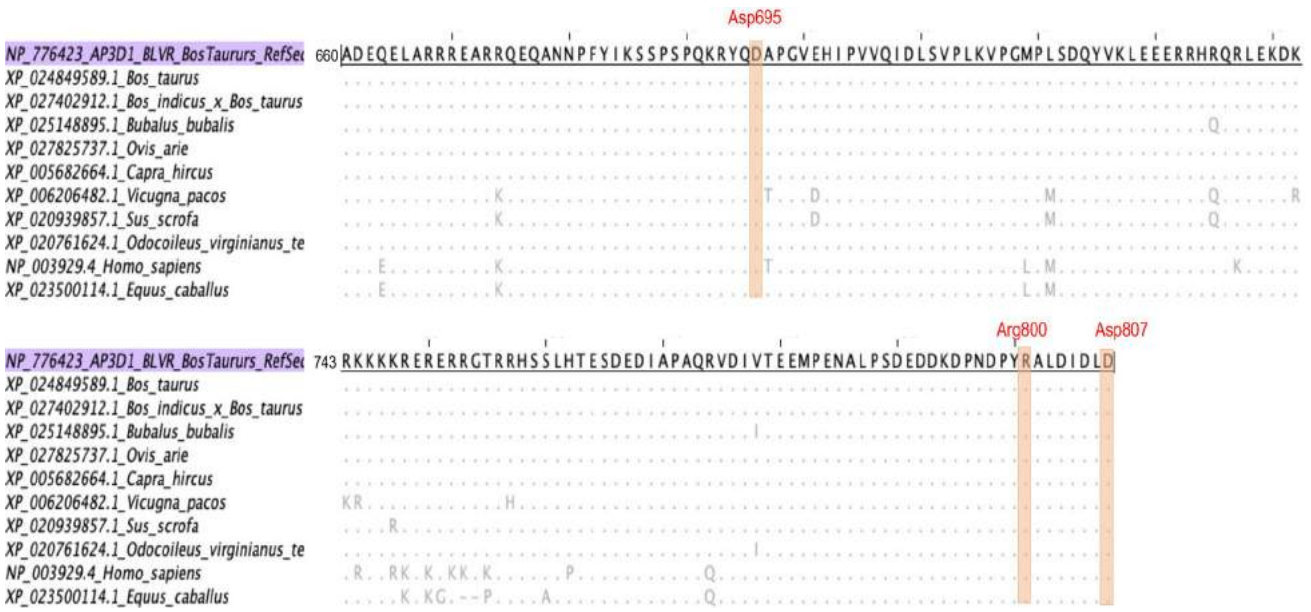

Fig. 2 ClustalW multiple alignment of AP3D1 sequences of mammals, including ungulate animals and humans, using bovine AP3D1 as a reference. The region between aa 660 and aa 807 is shown, which belongs to the BLVR domain in cattle. The residues that interact with the BLV gp51 protein are highlighted in red. These amino acids were conserved in all species. Sequence variations were identified outside the range of the residues of interest for the interaction with gp51.

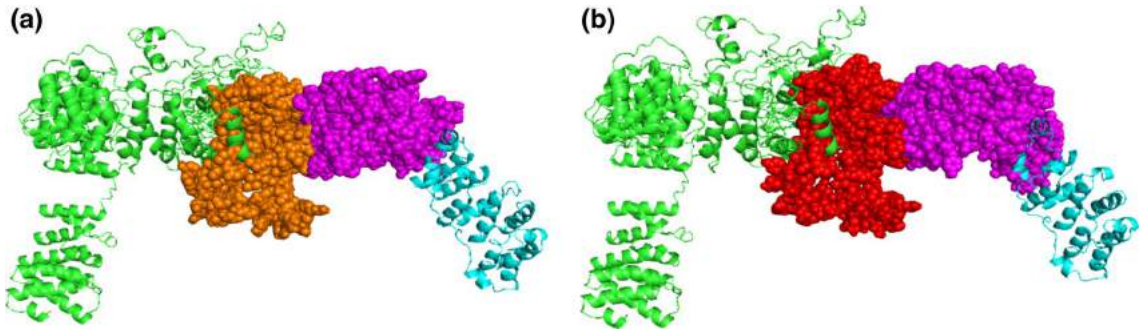

Fig. 3 Molecular docking simulations of AP3D1 and gp51 proteins. The AP3D1 protein is shaded in green for both species. The BLVR domain is highlighted in orange for sheep (a) and in red for buffalo (b). The Env protein is shaded in cyan, and the gp51 domain is highlighted in magenta. For both species, interactions between AP3D1 and the Env protein were predicted to involve the BLVR domain and gp51.

Table 2 BLV detection in sheep and buffaloes in different regions of Colombia

Species	BLV detection by department			BLV total* n (%)
	Antioquia n (%)	Cundinamarca	Santander	
Buffaloes (n = 61)	5 (8.19)	7 (11.4)	--	12 (19.67)
Sheep (n = 44)	--	9 (20.45)	6 (13.63)	15 (34.09)

The results are shown as frequencies and percentages for each region – *n (%)*

*BLV total represents cumulative frequency and percentage per species

were -395.7 +/6.2 and -11.9 kcal/mol, respectively, for sheep AP3D1 and -387.8+/-7.6 and -10.5 kcal/mol, respectively, for buffalo AP3D1.

Evidence of BLV in sheep and buffaloes in Colombia

BLV was detected in both sheep and buffaloes located in the three departments included in the study. Frequencies per species and region are shown in Table 2. BLV was detected in 15 of the 44 sheep samples and in 12 of the 61 buffalo samples, representing a positivity rate of 25.7% overall for the samples included in the study.

For phylogenetic analysis, positive samples with the highest concentration of the PCR product were selected for sequencing. The resulting sequences (sheep, n = 3, buffaloes, n = 4) were analyzed using BLASTn and found to be more than 99% identical to BLV sequences from Colombian cattle. In the phylogenetic reconstruction (Fig. 4), sequences were evenly distributed among reference sequences and other Colombian sequences obtained from cattle and cattle-derived food products. No specific branches were identified for BLV detected in sheep or buffaloes.

Discussion

Emerging infectious diseases are a topic of concern from the One Health point of view, which proposes management of infectious diseases as part of a whole system that includes animal, human, and environmental health, due to the interactions between species and the environment, favoring transmission and spread of microorganisms that can adapt to novel hosts or ecological niches [53, 54]. Particularly for potential zoonotic infections, adaptation profiles, spillover events, evolutionary fitness, and susceptibility of new potential hosts to infections should be considered, and strategies are needed for integrated management, prevention, and control of infectious diseases [55]. One of the concerns for microorganisms crossing the species barrier, especially viruses, is the interaction with cellular receptors that are present in multiple animal species, increasing the range of infection in nature [10, 35]. In this study, the AP3D1 receptor of BLV was analyzed, a transporter protein that is present in multiple eukaryotic species, including mammals [56]. The results of this study showed a high degree of conservation in this protein among cattle, sheep, and buffaloes (Fig. 2), particularly in the BLVR domain, from aa 660 to 803. In the docking simulation (Fig. 3), it was found that the AP3D1 protein of sheep and buffaloes was predicted to be able to bind to the gp51 protein of BLV with no differences in the binding domain compared to that predicted for bovine AP3D1 [36], supporting the hypothesis of the natural infection with BLV in these species, as was also supported by the HADDOCK scores and binding affinity predicted in the analysis, which were similar for both species. These interactions between AP3D1 of different species and BLV-gp51 would be expected to allow a broader host range for the virus, and this could explain the presence of BLV in multiple species. Evidence of use of the same receptor for cross-species infections has also been reported for other viruses,

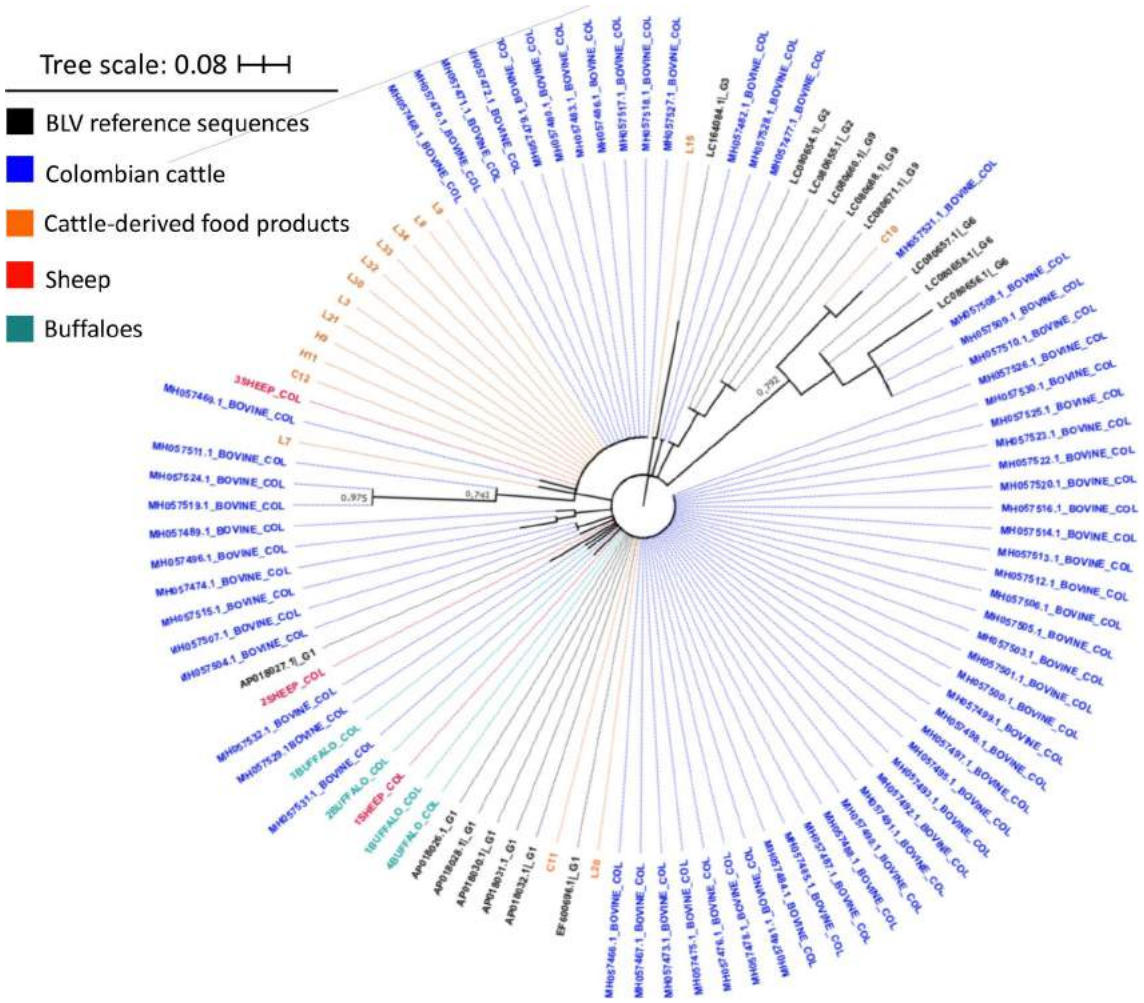

Fig. 4 Unrooted maximum-likelihood phylogenetic tree obtained using a multiple alignment of the complete data set (n = 104 sequences) produced using ClustalW and the Tamura-Nei model [52]. A region of the *tax* gene is shown. A discrete gamma distribution was used to model evolutionary rate differences among sites: 5 categories, +G parameter = 0.4839. Colors indicate the source of the BLV sequence as follows: black, BLV reference sequences; blue, cattle (blood); orange, cattle (food products, milk and beef); red, sheep; teal, buffaloes

including those with zoonotic potential such as influenza virus [57] and the current pandemics SARS-CoV-2 [58].

BLV has classically been studied in cattle regarding its implications for productivity, animal health, and the economic losses it causes for farmers and cattle keepers [17]. Although most infected animals remain asymptomatic, one of the biggest concerns is the spread of the virus among herds, which has been seen in the cattle industry, with high prevalence rates around the world, mainly in major cattle-producing countries [14]. Generally, in Colombia and in Latin America, cattle are slaughtered at an early age, except for females used for milk and reproduction purposes. For meat purposes, cattle are slaughtered between 2 and 3 years of age [41]. Lymphoma/leukemia development tends to occur in older cattle 3 years of age or more [59, 60]. Although BLV often remains undetected due to the removal of asymptomatic infected animals from the herds at an early age, the circulation and persistence of an oncogenic virus among animals has consequences in the long term, affecting animal health and food quality.

The results of this study suggest that BLV infects other species in regions of Colombia where it has been detected in cattle [40]. Few strategies are available for the prevention and control of BLV [61]. As no vaccines are available, prevention and control strategies are limited to managing risk factors associated with the transmission of the virus, such as use of disposable materials (e.g., syringes, needles, gloves), cleaning and disinfection of utensils, and segregating and culling infected animals. These strategies are useful for preventing the spread of infection [17, 62] but are not always implemented in the livestock industry. The detection of BLV in multiple species increases the challenge of management

and control of the viral infection and monitoring its dissemination and transmission patterns. Good veterinary practices should be considered for improving animal health and preventing cross-infections, particularly in mixed-species herds.

One of the biggest concerns in Latin America and the Caribbean is the lack of support by governments for diagnosis, and no budget is available for this purpose. In Europe, eradication programs have been established with successful results in at least 22 countries through rigorous surveillance programs financed by the government [63–66]. Chile is the only country in Latin America in which epidemiological surveillance has been implemented and an eradication program has started [67]. Efforts to establish eradication programs for the elimination of BLV should be made in the livestock industry as a prevention and control strategy whose impact will be seen in the future. Improvements in diagnosis and surveillance programs are necessary as a basis for follow-up of BLV infections in cattle as well as other intermediate hosts in which the virus could be spreading [68].

In Colombia, sheep and buffaloes are increasing in importance in the livestock industry and are commonly present on mixed-species farms, which have significantly increased in number in the last decade. Now, although the Instituto Colombiano Agropecuario (ICA) [69] exists as a regulatory party within the country and oversees animal health and surveillance programs, follow-ups for these animal species are limited and should be improved in order to gain better and broader coverage in terms of detection and control of infectious diseases. BLV is included in the list of viruses for which reporting is mandatory, which means that farmers/laboratories that perform voluntary diagnosis and detect the virus within their herds are required to report the presence of the virus to the government [45], but since it is not considered an officially controlled disease, it is not the responsibility of the government to perform diagnosis and verify that herds are BLV-free, as is the case with foot-and-mouth disease, rabies, tuberculosis, and brucellosis [70]. The lack of control of infections in buffaloes, sheep, and cattle facilitates the dissemination of BLV through livestock trading and commercialization.

There have been few reports of the presence of BLV in sheep and buffaloes in other regions, but it is becoming an important topic in the livestock industry worldwide. In Latin America, a previous study in Venezuela showed that BLV was present in buffalo milk on about 27% of farms tested in Maracaibo (n = 22) [71], while in the southeast and Amazon regions of Brazil, BLV was not detected in a study that included 300 buffaloes [72]. In contrast, a study in Egypt found a seroprevalence of 9% in buffaloes on mixed farms with cattle and camels [20], and in Pakistan, the virus was found in 0.8% of analyzed samples [73]. Antibodies against BLV were detected in sheep in the state of Sao Paulo in Brazil, although the incidence was very low, with only two out of 2592 sheep sera testing positive [24]. In Iran, the presence of BLV in mixed herds of cattle, sheep, and camels was investigated, and it was found in sheep (5.7%) and cattle (23%), but not in camels [23].

Since there was no information available about the presence of the virus in species other than cattle in Colombia, we used a convenience sampling strategy for the screening of the virus in buffaloes and sheep. The frequency of detection (11% in buffaloes and 20% in sheep) was higher than in other geographical regions. Although it is not known whether similar BLV infection rates will be found in other departments where buffaloes and sheep are located, these results can be used as a basis for further epidemiological studies with broader populations of animals to determine the current prevalence rates of BLV in Colombia, especially on mixed-species farms. Phylogenetic analysis (Fig. 4) performed with the sequences obtained from sheep and buffaloes in the current study and previously reported sequences from Colombian cattle, suggested that the circulating virus could be the same as that obtained from cattle, since no separate branches were identified in the phylogenetic tree for sequences obtained from sheep and buffaloes [40].

The presence of BLV in multiple species, its high prevalence rate (62%) in cattle in Colombia [40], and the potential for co-circulation of the virus in cattle and humans [33] should raise concerns for regulatory agencies and livestock producers, as BLV infections are unfavorable for the animal production sector [16, 17], and the zoonotic potential of the virus has implications for human health [31, 74]. Accumulating evidence in the literature suggesting that BLV can infect multiple species should raise concerns about elevated dissemination rates on mixed-species farms, in wildlife reservoirs, and in accidental hosts, which could hamper prevention and control strategies to stop the spread of the virus. Efforts should be taken towards the development of new strategies for follow-up of the virus and the implementation of eradication programs similar to those used in Europe so that in the future, herds worldwide will become BLV-free as part of the One Health initiative [54].

Conclusions

We found evidence of BLV circulation in sheep and buffaloes in Colombia, with 25.7% positivity in the animals tested. Also, the potential interaction of the AP3D1 proteins of these species with the BLV gp51 protein suggest that AP3D1 could be used as a receptor for BLV in buffaloes and sheep, which might play a role in the dissemination of the virus on mixed-species farms, potentially complicating prevention and control strategies and surveillance programs.

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Declarations

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6.3. Evidencia de marcadores moleculares del VLB compartidos entre humanos y bovinos: determinación de haplotipos circulantes

Como parte de las evidencias de las infecciones zoonóticas, los análisis desde la epidemiología molecular han permitido identificar las dinámicas de transmisión y diseminación que ocurren entre los patógenos y sus hospederos así como visualizar eventos de intercambio genético, mutaciones y adaptaciones que pueden tener estos patógenos al lograr cruzar las barreras entre especies (117). Determinar en su totalidad las vías de transmisión y de diseminación de las infecciones zoonóticas es un reto debido a los procesos complejos de propagación, la dificultad en el diagnóstico y seguimiento de brotes epidemiológicos específicos y a su vez la caracterización de los patógenos circulantes y sus correspondientes subtipos o variantes (118,119).

Para entender las dinámicas de transmisión de estos patógenos, más allá de identificar las variaciones puntuales que puedan ocurrir en cada individuo infectado, es importante analizar los marcadores moleculares a nivel poblacional para poder entender algunos factores de ecología microbiana e identificar si se encuentran variantes predominantes que puedan estar asociadas con la patogénesis, contagio o transmisión de estos microorganismos, o incluso los polimorfismos predominantes a nivel poblacional (120). Los análisis poblacionales de diversidad génica pueden incluso aportar aspectos o patrones de diseminación específicos para cada comunidad que pueden pasar desapercibidos con los análisis individuales como principios de susceptibilidad genética o de resistencia a medicamentos (121).

Con respecto al VLB, debido a que es poca la evidencia del seguimiento del virus en otras especies diferentes a los bovinos, no existen análisis comparativos que involucren múltiples comunidades o poblaciones para entender las dinámicas de transmisión del virus. La mayoría de los estudios de epidemiología molecular se enfocan en la identificación de los genotipos circulantes del virus en el ganado bovino a nivel mundial. Hasta el momento, se han descrito 10 genotipos para el virus aunque no existe correlación con patrones de patogenicidad, de evolución de la enfermedad ni de perfiles de transmisión (9). En Bolivia se identificó un genotipo específico de esta región (G9), con mutaciones puntuales debido a condiciones de barrera geográfica de ese país (122).

Con los resultados presentados en las secciones anteriores de este documento se ha evidenciado la presencia del virus en muestras provenientes de diferentes fuentes como los bovinos, mujeres con y sin cáncer, productos alimenticios derivados de bovinos como carnes y leches, y en otras especies animales como los búfalos y las ovejas. En el análisis de prevalencia realizado en Colombia, reportamos los genotipos 1 y 6 del virus en el ganado vacuno distribuido en las principales regiones productoras del país (Anexo 1). En el caso de los búfalos y las ovejas, se comparó un segmento del gen *tax* con secuencias de genoma completo del virus previamente reportadas en el GenBank, en donde se confirmó la identidad del virus por análisis de BLAST y se realizó un análisis filogenético de máxima verosimilitud en el cual no se identificaron ramificaciones específicas para las muestras provenientes de estas especies, sugiriendo que se trata del mismo virus circulante en el ganado vacuno (Anexo 5). Para los humanos y los productos alimenticios (anexo 2 y 4), se utilizó un fragmento del gen *gag* como marcador molecular para el diagnóstico y la secuenciación debido a que este fragmento es considerado como una de las regiones más conservadas del virus (1), para las cuales también fue posible confirmar la identidad del virus con la herramienta BLAST.

Al tener las secuencias de *gag* obtenidas a partir de bovinos, humanos y alimentos, se realizó un análisis comparativo desde la epidemiología molecular con el fin de reconstruir las redes de transmisión que pudieran estar ocurriendo entre las interfaces de los humanos y animales para el caso de Colombia (artículo 5). Inicialmente se realizó un análisis de diversidad génica para identificar los haplotipos circulantes a nivel poblacional. Posterior a esto, se realizó un análisis filogenético de máxima verosimilitud para identificar si se presentaban ramificaciones específicas por los tipos de muestras analizadas, seguido de un análisis de redes de haplotipos y de eventos de recombinación entre las fuentes de obtención de las muestras. Por último, se analizaron los factores de riesgo que podrían estar implicados en la población de mujeres para la adquisición del virus, según los factores a los que estuvieran expuestas las participantes como el contacto directo con el ganado bovino, contacto con el entorno o el consumo de alimentos derivados de bovinos como la leche, la carne y derivados lácteos de preparación artesanal.

Con estos resultados se pudieron evidenciar patrones moleculares como indicios de un flujo genético entre los bovinos y los humanos, aportando a la hipótesis del potencial zoonótico del virus

ya que con los análisis realizados se pudo observar que no hay un tipo de virus exclusivo del humano y que, por el contrario, la presencia de los haplotipos se distribuye de manera heterogénea entre las fuentes analizadas, brindando evidencia de la presencia del virus en las interfaces de contacto entre los bovinos y los humanos, así como eventos de reticulación que indican un intercambio genético entre las fuentes analizadas. Adicional a esto se encontró una correlación entre la presencia del virus en los humanos y el consumo de leche cruda y derivados lácteos de fabricación artesanal, lo que propone a los alimentos como potenciales fuentes de diseminación. Estos resultados permitieron avanzar en el conocimiento de las dinámicas de transmisión que pueden estar ocurriendo para el VLB en los humanos, fortaleciendo la hipótesis de su procedencia desde los bovinos, aportando una evidencia más para afirmar que se trata de una infección de tipo zoonótica.

Teniendo en cuenta que el virus se distribuye a nivel mundial con tasas de prevalencia altas en la mayoría de países de América Latina (9) así como las altas tasas de consumo de alimentos derivados de bovinos, la identificación de estas dinámicas de transmisión genera una alerta para plantear estrategias de prevención y control adecuadas desde la perspectiva de una "Una Salud" (93), incluyendo aspectos de seguridad alimentaria, mejoras en la salud animal y así, de manera indirecta, prevenir el ingreso de este virus a los humanos, como ya ocurre en algunos países de Europa (10,11,13). La presencia del virus en múltiples hospederos hace ver la necesidad de realizar un manejo integrado de buenas prácticas pecuarias para garantizar buenos estándares de salud animal seguidos de la cadena de producción de alimentos, para garantizar que el humano, quien es el consumidor final, obtenga productos de buena calidad que no pongan en riesgo su salud (90).

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Co-Circulation of Bovine Leukemia Virus Haplotypes among Humans, Animals, and Food Products: New Insights of Its Zoonotic Potential

Adriana P. Corredor-Figueroa [1,2,†], Nury N. Olaya-Galán [1,3,*,†], Sebastian Velandia-Álvarez [1], Marina Muñoz [4], Sandra P. Salas-Cárdenas [1], Milcíades Ibáñez-Pinilla [5], Manuel A. Patarroyo [6,7,8] and Maria F. Gutiérrez [1]

1 Grupo de Enfermedades Infecciosas, Laboratorio de Virología, Departamento de Microbiología, Pontificia Universidad Javeriana, Bogota 110231, Colombia; acorredorf@ecci.edu.co (A.P.C.-F.); velandia.s@javeriana.edu.co (S.V.-Á.); sandra.s909@gmail.com (S.P.S.-C.); mfgutier@javeriana.edu.co (M.F.G.)
2 Vicerrectoría de Investigación, Universidad ECCI, Bogota 111311, Colombia
3 PhD Program in Biomedical and Biological Sciences, Universidad del Rosario, Bogota 111221, Colombia
4 Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogota 111221, Colombia; claudia.munoz@urosario.edu.co
5 Research Department, Hospital Universitario Mayor Méderi—Universidad del Rosario, Bogota 111411, Colombia; milciades.ibanez@urosario.edu.co
6 Molecular Biology and Immunology Department, Fundación Instituto de Inmunología de Colombia (FIDIC), Bogota 111321, Colombia; mapatarr.fidic@gmail.com
7 Microbiology Department, Faculty of Medicine, Universidad Nacional de Colombia, Bogota 111321, Colombia
8 Health Sciences Division, Main Campus, Universidad Santo Tomás, Bogota 110231, Colombia
* Correspondence: nury.olaya@urosario.edu.co; Tel.: +57-3002081160
† These two authors contributed equally as first authors.

Abstract: Bovine leukemia virus (BLV) is the causative agent of leukemia/lymphoma in cattle. It has been found in humans and cattle-derived food products. In humans, it is described as a potential risk factor for breast cancer development. However, the transmission path remains unclear. Here, a molecular epidemiology analysis was performed to identify signatures of genetic flux of BLV among humans, animals, and food products. Sequences obtained from these sources in Colombia were used (n = 183) and compared with reference sequences available in GenBank. Phylogenetic reconstruction was performed in IQ-TREE software with the maximum likelihood algorithm. Haplotype (hap) distribution among the population was carried out with a median-joining model in Network5.0. Recombination events were inferred using SplitsTree4 software. In the phylogenetic analysis, no specific branches were identified for the Colombian sequences or for the different sources. A total of 31 haps were found, with Hap 1, 4, 5 and 7 being shared among the three sources of the study. Reticulation events among the different sources were also detected during the recombination analysis. These results show new insights about the zoonotic potential of BLV, showing evidence of genetic flux between cattle and humans. Prevention and control strategies should be considered to avoid viral dissemination as part of the One Health program policies.

Keywords: molecular epidemiology; bovine leukemia virus; haplotypes; zoonoses; recombination analysis

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1. Introduction

Zoonoses are described by the World Health Organization and the Centers for Disease Control and Prevention as any disease or infection transmitted naturally within the human–animal interface (e.g., direct contact with animals and its environment, animals' body fluids, animal-derived food products), leading to the emergence of new or previously unknown infections in humans [1,2]. Zoonotic infections have a special impact in developing countries, due to a higher exposure to risk factors such as contact with

animal sources, low hygiene conditions and contamination of water sources [3,4]. One of the major threats for these infections is through the consumption of unindustrialized, undercooked, and raw food products mainly obtained from livestock or poultry, which could be infected or contaminated with pathogens [5]. Determining the complete pathway of zoonotic infections is challenging due to the several steps that are needed for spillover of infections and epidemiological detection of specific outbreaks as well as the identification and characterization of circulating pathogens [6].

Bovine Leukaemia Virus (BLV) is a retrovirus within the *deltaretrovirus* genus; is included within the five most important viral agents in livestock production and is the causative agent of enzootic bovine leukosis, which is a lymphoproliferative chronic disease which mainly affects cattle herds. Most of the infected cattle (70%) remain without any clinical signs, while some of them evolve into leukemia and/or lymphoma (10–15%) after a persistent infection over several years [7]. BLV is distributed worldwide with prevalence rates between 5 and 90% among different regions [8], with a particular impact both in North and Latin American countries with the highest reported prevalence rates [9–12].

Besides cattle, some other animal species are also susceptible to infection by BLV, including buffaloes, sheep, goats and alpacas [13–17]. BLV has been proposed in the literature as a pathogen with the capability to infect multiple species. This has been shown both naturally and experimentally, demonstrated by BLV's capacity to induce leukemia/lymphoma in experimental animals and its capacity to infect multiple cell lines obtained from different sources [18–20]. Thus, the hypothesis of trespassing the species-specific barrier in natural environments has been proposed, supported by the broad range of mammals that BLV can infect, possibly through shared receptors between species [21,22]. The presence of the virus in different species as part of the natural course of BLV infection makes it difficult to implement and execute prevention and control strategies in livestock production, favoring transmission processes in mixed herds (e.g., sheep, goats, and cattle).

In addition, evidence of BLV in human beings has been reported by different researchers via the presence of its gene segments, proteins and antibodies against the virus in different parts of the world [23–28]. Epidemiological studies have identified a significant association between the presence of the virus and breast cancer development, proposing BLV as an intermediate risk factor for cancer outcome [29,30]. Furthermore, evidence of BLV and some other viruses has also been reported in lung cancer, showing a correlation with the up and down regulation of metabolic pathways associated with cell control and oncogenesis [31,32]. Even though the presence of the virus in humans has been reported, further studies are needed to completely understand its mechanisms related with oncogenesis as well as its transmission pathways in this specific population. Hence, there are still a considerable knowledge gaps as regards the interactions of animal species and human beings.

In cattle, the transmission pathways are well characterized, involving: processes of direct contact between body fluids; transmission through iatrogenic procedures, such as vaccination; dehorning and insemination processes [7]. Moreover, vertical transmission by breastfeeding from cows to calves has also been reported. Nonetheless, these transmission pathways between species and into humans are not clear, but it has been hypothesized that transmission to humans occurs through the consumption of animal-food products that might be infected with the virus [27].

Previous studies by our research group have found the presence of the virus both in cattle [10] and humans [33], as well as the presence of viral gene segments in raw beef and milk [34]. However, no concluding remarks have been described towards the proposal of a zoonotic infection from cattle to humans, hence, the transmission pathway has not been clarified. In order to advance the knowledge of BLV's transmission patterns, this study was aimed at understanding the transmission profiles of the virus when it is present in different sources (cattle, humans, and food products), through a comprehensive phylogenetic analysis between sequences obtained from the above-mentioned sources

as well as by identifying recombination events between viral isolates, and risk factors of exposure in the human population.

2. Materials and Methods

2.1. Population and Samples

Samples stored at −20 °C at the Virology Lab of the Pontificia Universidad Javeriana obtained between 2015–2018 and collected from cattle blood [10], human female breast tissues and blood [33] and cattle-derived food products (milk and beef) [34] from different regions of Colombia were used in this study. In these samples, presence of BLV had previously been detected, positive samples were sequenced and deposited in GenBank.

Briefly, total DNA extraction was performed using High Pure PCR Template Preparation Kit (Roche Applied Science®, Mannheim, Germany) following the manufacturer's instructions. BLV detection was carried out via PCR amplification, with the PCR Master (Roche Applied Science®, Mannheim, Germany) targeting the *gag* region of the virus (nt 1068–1453; PF–AACACTACGACTTGCAATCC; PR–GGTTCCTTAGGACTCCGTCG). To increase the sensitivity of the PCR, in some cases (mainly female samples and food products), a nested PCR was performed (nt 1097–1369) [27]. Subsequently, positive samples of BLV for this region were sequenced using Sanger technology in Macrogen Inc. (Seoul, Korea) using the same two primers used to detect the viral presence and the final sequences were deposited in the GenBank repository for further analyses.

2.2. Data Retrieval and Viral Target Region

For the molecular phylogenetic analysis, Colombian sequences from the three sources were downloaded from GenBank (cattle—MH293473.1 to MH293501.1, humans—MN831896 to MN831962, and food products—MH057402.1 to MH057465.1) as well as some other reference sequences. A total of 259 sequences were included in the analysis. Of these, 64 of them were obtained from the peripheral blood of cattle, 67 were obtained from breast tissue and the blood of Colombian females with and without breast cancer; 29 were from cattle-derived food products; 27 were obtained from women breast tissue; finally, 72 reference sequences of the complete BLV genome—obtained from cattle around the world—were used. Complete BLV genome sequences available in GenBank were used as the reference sequences. Supplementary Tables S1 and S2 show the details of the sequences included in the study.

Initially, Colombian sequences were aligned using MAFFT (v7.427—2019, available at https://mafft.cbrc.jp/alignment/software/ (accessed on 27 March 2021), Kyoto, Japan), under the automatic settings of the program [35,36]. After alignment, a diversity analysis of the Colombian sequences was carried out in DNAsp [37] to select the dataset with the most informative region within the *gag* gene.

A region of 182 base pairs was selected for further analyses, considering the region with the greatest number of haplotypes within the analyzed population and the least number of loses due to the quality of Sanger sequencing in the extremes of the sequences. Later, the same frameshift was filtered in the reference sequences of BLV sequences obtained from GenBank.

2.3. Phylogenetic Analyses

After multiple alignment of the complete data set was obtained, a phylogenetic reconstruction was conducted in IQ-TREE software multicore version 1.6.12 [38]. A previous selection of the most relevant nucleotide substitution model in ModelFinder [39] was carried out with 1000 ultrafast bootstrap replicates using UFBoot2 [40]. Branching support metrics with aLRT [41] and its nonparametric equivalent SH-aLRT [42], with 1000 replicates, were also considered. The final edition of the phylogenetic tree was carried out in the ITOL program available online (https://itol.embl.de (accessed on 25 June 2020)).

2.4. Haplotype Distribution and Network Analysis

The haplotypes in the evaluated dataset were identified with a Fasta matrix, constructed for haplotype network analysis in the DNA alignment software (Fluxus Technology Ltd., Colchester, UK, available at http://www.fluxus-engineering.com/align.htm (accessed on 27 March 2021)). In parallel, the median-joining model, based on 1000 iterations with default parameters in Network 5.0 software, was used (Fluxus Technology Ltd., Colchester, England, available at http://www.fluxus-engineering.com/sharenet.htm (accessed on 27 March 2021), [43,44]).

2.5. Identification of Recombination Events between Colombian Isolates Obtained from Cattle, Food Products and Humans

After haplotypes in the data set were identified, recombination analyses were performed to identify the molecular rearrangements among the sequences, represented by reticulation events among different sources. For this purpose, a representative sequence for each haplotype was selected and the consequent haplotype alignment was used as the input for the phylogenetic networks. Analyses were performed using the Neighbor-Net method [45], available in the SplitsTree4 package (Version 4.14-4, Tübingen, Germany) with 1000 iterations. Finally, recombination indexes were determined with DNAsp (v.5.0, Barcelona, Spain) as markers of genetic diversity in the analyzed population based on the haplotypes detected.

2.6. Identification of Risks of Exposure to BLV in Humans and Association with Circulating Haplotypes

Human samples were taken from a cohort of patients between 2016 and 2018 with benign and malignant breast tumors, being treated at the breast surgical service at Méderi Hospital (MH) located in Bogotá, Colombia. In addition, samples were also collected from a secondary population of deceased females without tumor development on the breast.

The study was approved by the ethics committee of Universidad del Rosario (UR) and Méderi Hospital (Record No. CEI-ABN026-000 241, 2016). All procedures were performed in accordance with the ethical standards of the institution and with the 1964 Helsinki declaration and its later amendments (last revision 2013). All the participants or relatives (in the cases of deceased females) voluntarily signed an informed consent form prior to sample collection. Data obtained during the study was used under confidentiality.

After the informed consent was signed, a survey of the participants/relatives was conducted to collect information regarding possible exposure factors related with the acquisition of BLV from cattle (i.e., direct contact, living in shared environments, contact with blood of cattle, and consumption of cattle-derived food products including meat, dairy products, and raw milk). As for deceased females for whom the quality of information was not accurate and incomplete, missing data were excluded from the analysis. A risk assessment was performed for patients from Mederi Hospital, for whom an association between the presence of the virus and breast cancer had previously been identified [33].

Chi-square bivariate analysis was performed to identify if any of the above-mentioned factors were significant for the presence of BLV in humans. Afterwards, a Mann–Whitney analysis was performed to detect the effect of multiple variables in the model, followed by a multinomial logistic regression to identify Odd Ratios (OR) values related with the acquisition of the virus. Finally, a chi-square bivariate analysis was performed between the obtained haplotypes in humans and the exposure factors obtained in the study. Results were considered statistically significant if they had a p value of <0.05 with a confidence interval of 95%. Statistical analyses were performed in SPSS (Ver. 25.0, IBM Corp., Amonk, NY, USA) and STATA (Ver. 15, StataCorp LP, College Station, TX, USA).

3. Results

3.1. Phylogenetic Analyses

The comprehensive phylogenetic analysis showed common characteristics among the Colombian sequences of BLV obtained from cattle, food, and humans. In the phylogenetic

reconstruction with IQ-TREE, a Jukes–Cantor model was identified as the best substitution model (Figure 1). It was found that the virus obtained from different sources had a heterogeneous distribution, with no specific branches within the human, cattle, or food sequences. When compared with the sequences available in GenBank, mixed patterns were found among the Colombian human, food, and bovine sequences. From this, it can be inferred that the virus is circulating among the analyzed sources and does not generate specific clusters among the sequences of the data set due to its heterogeneous distribution.

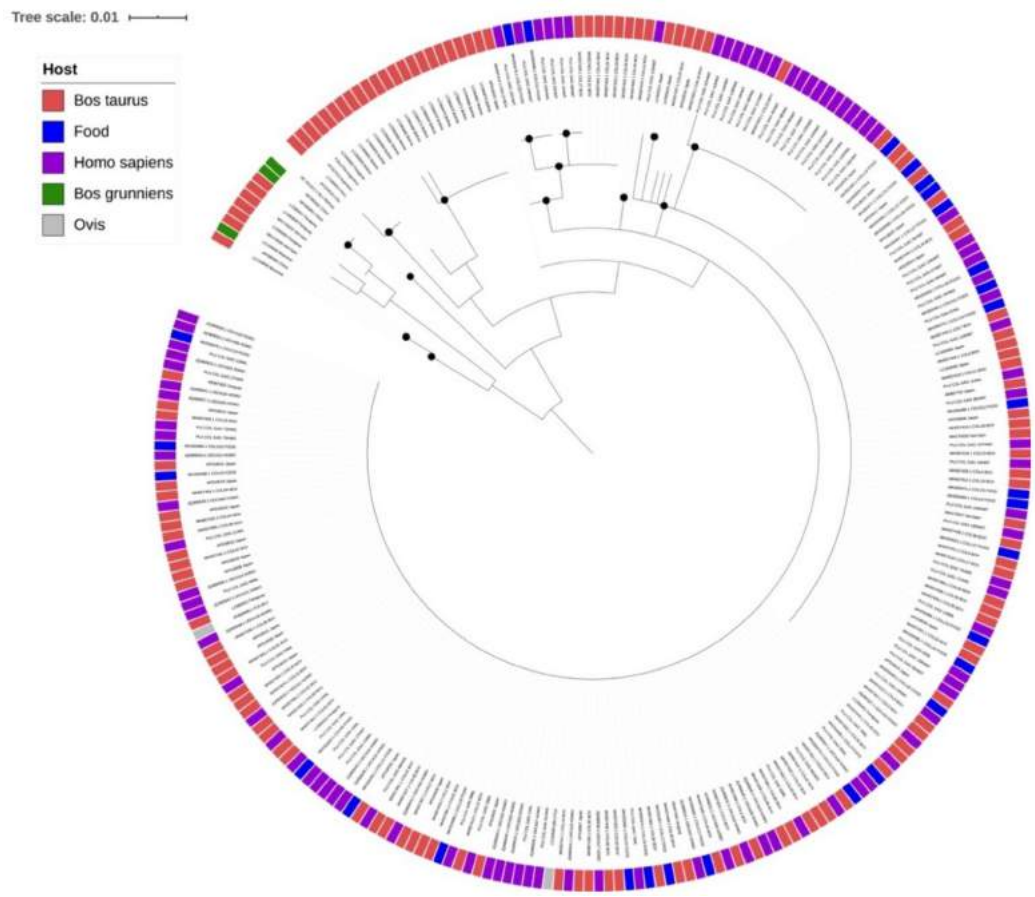

Figure 1. IQ-TREE phylogenetic reconstruction obtained from the multiple alignment performed in MAFFT of the complete data set (GenBank reference sequences and Colombian sequences). A region of 182 pb of the *gag* gene is shown. Colors indicate source of the virus. Red—bovine, Blue—food, Purple—humans. Jukes–Cantor substitution model was used in IQ-TREE. Black dots represent nodes higher than 90%, with a bootstrap of 1000 replicates.

3.2. Haplotype Distribution and Network Analysis

A haplotype network analysis (Figure 2) was carried out to evaluate the distribution patterns of BLV among the three populations, potential dissemination profiles and the eventual transmission networks between cattle and humans. Thirty-one haplotypes were identified (Hap 1–31) in the complete data set with a haplotype diversity of 0.7256. The most predominant haplotype was Hap 1 (n = 117) followed by Hap 4 (n = 66).

In the haplotype network, the Colombian sequences obtained from the three evaluated sources were mainly distributed in Hap1 and Hap4 (identified with green squares and arrows, Figure 2). Nucleotide changes among Hap 1 and 4 were shared between cattle, food, and humans. In Hap 1, sequences from Japan, Uruguay, and Paraguay were also found. In Hap 4, sequences from other countries including Japan, China and Vietnam were also found.

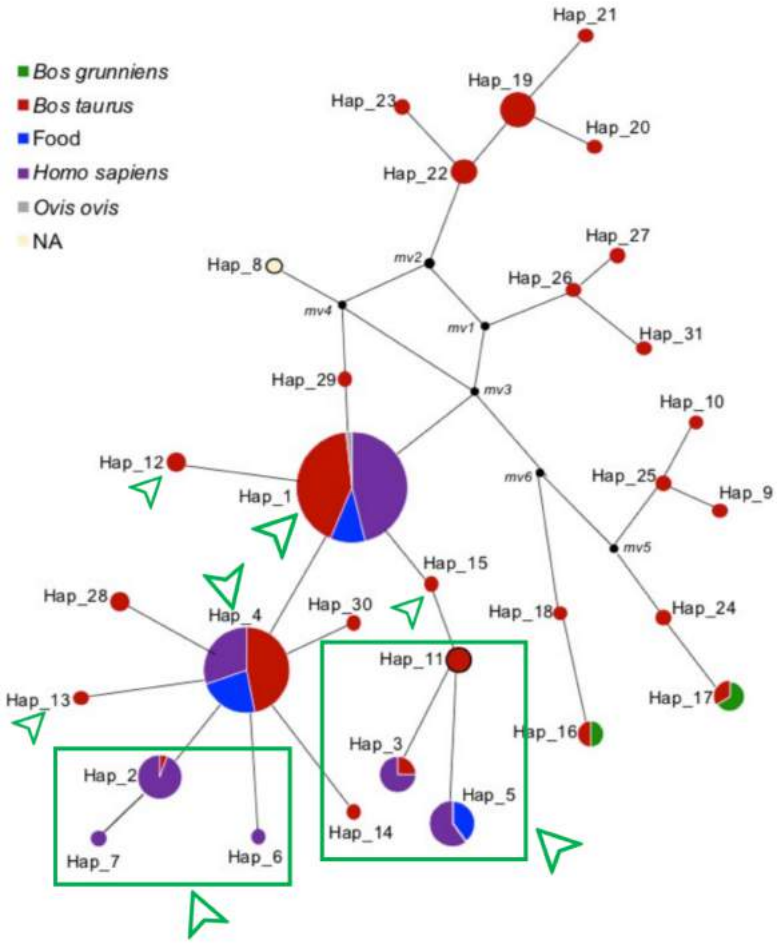

Figure 2. Haplotype network of Bovine Leukaemia Virus (BLV) obtained from the multiple alignment of the complete data set, performed with Median-Joining tool in Network v.5.0. A total of 31 haplotypes were identified in the analyzed dataset. The size of the circles represents the frequency of the haplotypes in the study. Hap 1 (n = 117) and Hap 4 (n = 66) were the most frequent haplotypes. Distances from haplotypes represent the sequence-type differences as regards nucleotide substitutions in the data set Colors indicate source of the virus. Red—bovine, Blue—food, Purple—humans. Notice the three colors in Hap 1 and 4. Small black circles were generated automatically to estimate connectors of the analyzed sequences. Haplotypes shown in green squares and arrows indicate those in which Colombian sequences were identified.

Haps 1 and 4 showed other variations such as those found in Haps 2, 7 and 6 for humans and Haps 19 to 23 in bovines. These haplotypes were more divergent than those shared among all the sources evaluated. Here, Haps 2, 3, 5–7, and 11–14 were exclusive to the sequences obtained from sources in Colombia. Shared haplotypes between sources of the virus were observed in Haps 2, 3 and 5, although no evidence of sequences from other countries was identified.

In the other haplotypes identified in the study, no sequences from Colombia were found but certain specific clusters by countries were visualized, as in the case of the Bolivian sequences in Hap 19. In Haps 28 and 30, which have a phylogenetic closeness to the Colombian nodes, sequences obtained from Japanese cattle were identified.

3.3. Identification of Recombination Events

The recombination analyses performed on the complete dataset in the SplitTree program (Figure 3) revealed reticulation events between the identified haplotypes, representing evidence of recombination signals. These reticulation events were identified at the inter-

sections of the network, in which sequences from cattle, humans and food are shared, evidencing the flow of genetic information between cattle and humans.

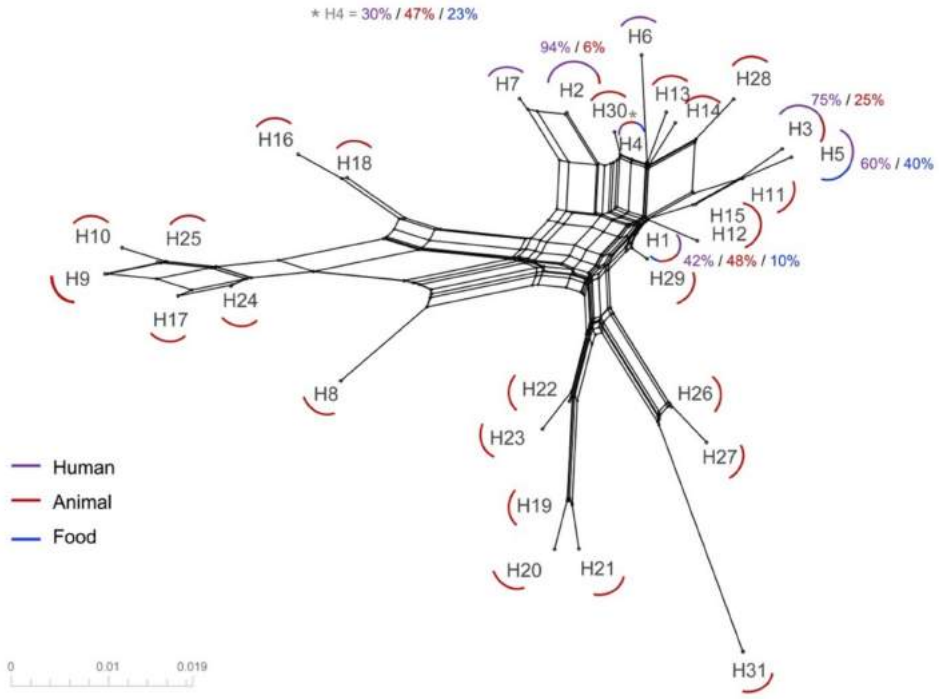

Figure 3. Phylogenetic network obtained from the alignment of representative haplotypes based on Neighbor-Net algorithm performed on SplitsTree. Reticulation events among haplotypes were identified, including both Colombian sequences and those from other regions. Colors represent sample source: Red—bovine, Blue—food, Purple—humans. Reticulation events between mixed sources were identified, particularly for Haps 1 and 4 at the central network hub, in which the three sources of the virus were identified.

Recombination events were mainly identified by crosslinking in the middle of the network, led by Hap 1 and Hap 4, and with the absence of specific branches for sequences of human origin. Several recombination signals were observed in the sequences, indicating that the fragment used has sufficient resolution power (genetic divergence markers) to compare the virus among sources.

A total of 36 polymorphic sites were identified, with a minimum of five recombination events determined in DNasp. The genetic diversity index showed a variance of the distribution of the samples (Sk ^ 2) of 9213, with a θ index of 2671 per gene ($R < 0.0001$) between adjacent sites. It should be noted that with these results, recombination events were found in the exclusive bovine haplotypes, which are associated with the genetic diversity in its natural host. Likewise, a flow of genetic diversity transmitted to humans was found, shared by reticulation events among the three sources.

Haps 1 to 7, which were found in humans, were also found in sequences obtained from bovines, three of which (Haps 1, 4 and 5) were from food products. In addition, the haplotypes with co-circulation among the three evaluated sample sources were associated with the most reticulation events, providing evidence of genetic exchange among haplotypes found in livestock, food, and humans, as a sample of the flow of genetic information between sources.

3.4. Risks of Exposure to BLV and Haplotypes Association

BLV was previously detected by our research group in women with different diagnoses (breast cancer, benign pathology of the breast, no tumor development), and a significant association was identified between the presence of the virus and breast cancer in the

Colombian female population [33]. Here, with the same basis population (n = 168) for cancer risk assessment, in which BLV was present in 61.3% of malignant samples and 46.5% of benign samples, we analyzed the potential exposure factors related to acquiring the virus independently of the diagnoses obtained in the participants. Deceased females (participants with no tumor development) were excluded from the risk analysis due to missing data retrieved from the relatives.

In the bivariate analysis, a correlation between the consumption of dairy products such as home-made natural yoghurt and flavored yoghurt as well as raw milk and number of dairy products were significant for the presence of the virus ($p < 0.05$). Age (≥50, <50) and city of origin of the participants (capital city or other) also showed significance for the analysis. Meat consumption, direct contact with cattle and body fluids, and living in shared environments with cattle were not significant for the study. Sociodemographic characteristics and risk of exposure regarding the presence of BLV are shown in Table 1. In the multinomial logistic regression, it was shown that females who had a higher consumption of dairy products, also had a higher risk of acquisition of BLV (OR = 2.424, CI 95%: 1.063–5.527, p = 0.035, Table 2).

Table 1. Sociodemographic characteristics of female participants and exposure factors to BLV. Bivariate analysis comparing the presence of the virus with the exposure factors.

	Viral Presence		
	Positive	**Negative**	***p* Value**
	***n* (%)**	***n* (%)**	
Pathology diagnoses			
Malignant samples (n = 75)	46 (61.3)	29 (38.7)	<0.001
Benign samples (n = 85)	41 (48.8)	44 (51.2)	
Age			
≥50	39 (63.9)	22 (36.1)	0.039 *
<50	61 (49.6)	62 (50.4)	
Socio-demographic characteristics			
Origin			0.036 *
Bogotá	61 (49.3)	62 (50.4)	
Other	30 (66.7)	15 (33.3)	
Educational level			0.785
Elementary school	23 (52.3)	21 (47.7)	
High School	34 (58.6)	24 (41.4)	
Vocational and professional studies	34 (53.1)	30 (46.9)	
Risks of exposure to BLV			
Dairy Products consumption			
Flavored Yoghurt	70 (59.8)	47 (40.2)	0.023 *
Home-made natural yoghurt (Kumis)	61 (60.4)	40 (39.6)	0.042 *
Cheese	83 (55.0)	68 (45.0)	0.614
Jelly foot dessert (Gelatina de pata)	41 (60.3)	27 (39.7)	0.148
Industrialized milk	90 (54.2)	76 (45.8)	0.708
Direct contact with cattle	39 (55.7)	31 (44.3)	0.428
Amount of dairy products and raw milk			0.04 *
4 or more	31 (72.1)	12 (27.9)	
3	18 (52.9)	16 (47.1)	
2	4 (30.8)	9 (69.2)	
None	2 (66.7)	1 (33.3)	

* Significant results in the bivariate analysis for the viral presence were considered statistically significant for a p value <0.05.

Table 2. Multinomial logistic regression of the risks of exposure and viral presence in the human population. Odd-ratio values adjusted by age.

Variables	Viral Presence		
	β	OR (95% CI)	*p* Value
Age			
≥50	0.794	2.212 (1.111-4.402)	0.024
<50	–	1.00 (Reference)	–
City of origin			
Other	0.800	2.224 (1.030-4.805)	0.042
Bogota	–	1.00 (Reference)	–
Milk consumption and dairy products [a]	–	–	0.037 **
Only milk or 1 dairy product	−0.990	0.372 (0.103-1.346)	0.132
Two dairy products/milk	0.000	1.00 (0.431-2.321)	1.000
Three or more dairy products/milk	0.885	2.424 (1.063-5.527) **	0.035 **
No consumption of dairy products	–	1.00 (Reference)	–

[a] Amount of consumption of dairy products and raw milk. Dairy products include fresh cheese, flavored or natural yogurt, "gelatina de pata" (traditional dessert made with cattle collagen and milk). ** Significant results obtained for acquisition factors and the presence of the virus in the human population. *p* values <0.05 were considered statistically significant for the study.

Analysis of the correlation of circulating haplotypes with the exposure factors in the human population did not show statistical differences ($p \geq 0.05$). Haplotypes of BLV were evenly distributed among female samples with different exposure factors. In females with consumption of milk, dairy products, and beef, were present all the haplotypes of BLV reported in the study. The frequencies of occurrences of haplotypes and exposure factors are shown on Supplementary Table S3.

4. Discussion

Thus far, the studies that have been carried out since the molecular characterization of the BLV have been focused, above all, on genotyping and characterizing the worldwide viral distribution, emphasizing its molecular epidemiology [8]. However, few studies have focused on the diversity of the virus, nor on the dispersion and circulation patterns that can occur in the natural course of infection, considering both cattle as its natural host, and the interaction with the environment and arrival at accidental hosts, such as sheep and humans. In the current study, BLV sequences obtained from cattle, food, and humans were analyzed with the purpose of understanding the flux and transmission dynamics that the virus might possess to reach humans.

Shared distributions of the virus were identified among these three sources of analysis, indicating that the presence of the virus in humans is not an isolated event. Indeed, on the contrary, the results showed the co-circulation of haplotypes among the different sources, exhibiting recombination signatures, indicating that a common origin is shared. For the first time, these findings demonstrate genetic exchange among heterogenous hosts in the molecular marker used. Hence, this supports the hypothesis of the existence of transmission routes in the same ecological niche between cattle and humans.

Contemplating the globality of this study, there is evidence of congruence between the analyses carried out, revealing the transmission networks of the virus between cattle and humans, potentially using food as a dissemination vehicle [34]. These findings support evidence provided by previous studies [27,29] suggesting the transmission of BLV to human beings based on the evidence of the virus in food products and the presence of the virus in the bovine population with high prevalence rates and its worldwide distribution [8,10]. Statistically significant results were also identified in the current study with the consumption of dairy products and raw milk in humans (Table 2), supporting the hypothesis of transmission through cattle-derived food products.

In terms of the zoonotic principles of transmission (direct or indirect contact with animals, their environment, derivates and body fluids) [3], it seems that—at least in the

evaluated population—direct contact with cattle does not represent a high risk. Rather, risk is more related with cattle and milk distribution for human consumption. In addition, our results also showed a statistical significance with the age and city of origin of female participants (Table 2). This could be explained by longer exposure periods in older women during their lives to different risk factors related with the acquisition of the virus, increasing the probability of being infected. On the other hand, the origin of the participants could be related with accessibility to food products and a better quality (e.g., industrialized milk, meat of selected and controlled cattle) as, in the capital city (Bogotá), there is a higher level of control in slaughterhouses compared with smaller cities and towns in the surroundings of Bogota or even other regions in the country. Even if most of the participants were from Bogotá and lived there, the results showed a significant difference as regards the presence of the virus in people who lived in or were from other regions. However, it would be interesting to perform similar analyses in other regions of Colombia as well as in other countries in order to elucidate the impact of cattle-derived food products and their quality in terms of transmission of the virus to the human population. In our study, fewer people from other regions were included compared to those from Bogota, and thus, it was not possible to determine the impact of the virus in each specific region.

In the haplotype network (Figure 2), shared mutations in Haps 1 and 4 between sources of the virus indicate that these are closely related sequences, proposing the co-circulation of BLV haplotypes between bovines and humans. In addition, Haps 1 and 4 showed sequences from different countries that were obtained from cattle, which indicates that these changes in sequences are not specific to the Colombian population but are common profiles of the virus that are also present in other regions. These findings suggest that the presence of the virus in humans came from viruses circulating in cattle, through a common point of dissemination, such as cattle-derived food products.

Although the information obtained in this study is not enough to understand the origin of the virus in Colombia, it is interesting to evaluate the phylogenetic relationships that these sequences have in the dissemination flux (Figure 2). Likewise, it is important to highlight that the nodes located in the upper right part of the graph belong to sequences of cattle from different countries, indicating that these mutations already existed in reported sequences of the GenBank. On the contrary, on the left side of the graph, exclusive human haplotypes (Haps 6 and 7) were identified for the Colombian sequences, however, it does not refer to a specific evolutionary profile in humans but probably this suggests missing data at the population level, restricting detailed monitoring of the flow of the virus from cattle to humans.

Considering that this is the first study aimed at comparing sequences obtained from humans, food, and cattle, it should be noted that no human sequences from other regions besides Colombia are available. The lack of these sequences does not allow one to obtain the complete panorama of the dissemination profiles of the virus. Additionally, it would be interesting to analyze these patterns among other populations and other genomic regions of the virus. It would be expected that haplotypes would be grouped per countries and sources, as in the case of Haps 1 and 4.

In the phylogenetic network (Figure 3), identification of reticulation events between the different sources is suggested. From these results, it could be inferred that the BLV present in cattle and humans contains recombination events that indicate the flux of genetic information among sources. Additionally, shared haplotypes between species indicate co-circulation of the virus between humans and cattle, as well as the presence of BLV in food products. From the reticulation events found in this study, a recombination process in cattle before arriving to human beings is expected to be occurring, as no evidence of human-to-human transmission has yet been reported for BLV. The results obtained in the current study could suggest that the virus presents a transmission pattern in heterogenous hosts.

In the study of diseases of zoonotic origin, examples of other viruses have been recorded in the literature. Rabies is a classic example of a zoonotic infection, although spillover into the human population is stopped [46]. Other cases, such as the current SARS-

CoV-2 pandemic, demonstrate the rapid spread of diseases among the human population after the virus gains entrance into human beings [47,48]. Additionally, evidence of zoonotic infections through the consumption of animal-derived food products has been described, as in the case of hepatitis E, through the consumption of meat products obtained from pork [49,50]. Even though different models have been studied for emerging zoonotic infections from animals, the ability of infectious agents to remain in human beings differs among the different viral agents. This is mainly due to the capacity of the virus to perform complete and successful cycles in accidental hosts, as well as the impact on spillover among humans [51].

In the case of BLV, considering its worldwide distribution [8] and the high rates of consumption of beef and milk in human beings, it is possible that the virus can trespass the species barrier, thus, reaching humans. Our study demonstrated sequences with more than 95% of identity between cattle, humans and food products, no specific branches for each source in the phylogenetic analysis, and evidence of the most frequent haplotypes among the female participants. Haplotypes were equally distributed between the possible exposure factors for acquiring the virus in humans, and a significant correlation with the consumption of raw milk and dairy products was also found. Identification of these dynamics of transmission in BLV could guide the process of generating prevention and control strategies focused on its natural hosts, accidental hosts, reservoirs and ecological niches [3]. Even though further studies are still needed in order to elucidate the transmission factors for BLV infection to humans, prevention and control strategies should be considered to stop the viral spread worldwide, considering the One Health principle (humans, animals and environment as a whole) [52], as a result of the implications of the virus both in animal and human health.

5. Conclusions

In this study, it has been shown that BLV is circulating in humans, cattle, and food products in Colombia. Heterogenous distribution of the viral sequences was identified among sources, suggesting a transmission phase of the virus from bovines to humans based on the co-circulation of haplotypes among the evaluated sources. In addition, recombination signatures were detected in the phylogenetic networks. However, there is no evidence of human-to-human transmission, although the results obtained in this phylogenetic analysis suggest a genetic flux between the human–animal interface, probably through the consumption of infected food products.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijerph18094883/s1, Table S1: Accession numbers and details of the sequences included in the BLV analysis–*gag* region. Table includes reference and own sequences. Table S2: Sequences obtained in the current study from women samples collected from breast and blood. Population description., Table S3: Haplotypes distribution among humans positive to BLV regarding their exposure factors for viral acquisition. Chi-square bivariate analysis of exposure factors vs haplotypes.

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Data Availability Statement: Data is contained within the article or supplementary material. The data presented in this study are available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) as described in the 'Materials and Methods' section and Supplementary materials (Tables S1 and S2).

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7. EVIDENCIA EXPERIMENTAL DEL POTENCIAL ZOONÓTICO DEL VLB

Objetivo específico:

5. Aportar evidencia experimental de la capacidad zoonótica del virus a través de infección *in vitro* en líneas celulares humanas y análisis de su receptor celular.

La presencia de los patógenos en especies distintas a sus hospederos naturales es la principal evidencia del cruce de las barreras entre especies (68). Sin embargo, en los nichos ecológicos naturales de estos patógenos se generan muchos vacíos en el conocimiento lo que impide determinar en su totalidad las redes de diseminación y las dinámicas de transmisión de los patógenos en las interfaces de contacto de los humanos-animales, así como el impacto que tiene estas infecciones en los humanos (123). Para poder profundizar en el conocimiento de estos microorganismos con respecto a los mecanismos de acción, transmisión y patogénesis al atravesar las barreras de especie, es fundamental plantear modelos *in vitro* e *in vivo* bajo condiciones controladas para tratar de dar respuesta a factores que no se pueden resolver desde los nichos naturales de infección.

Los ensayos *in vitro* son una buena alternativa para estudiar el ciclo biológico de los virus y en la historia de la virología han permitido avanzar en el conocimiento de éstos y sus ciclos de infección (124). A través de estos ensayos se pueden determinar factores como la susceptibilidad de la infección en células blanco-específicas, la estabilidad de infección y evaluación de la progenie viral, así como el impacto que las infecciones virales puedan causar en las células. Por su parte, los ensayos *in silico* de biología computacional y bioinformática han permitido hacer uso de la información registrada en bases de datos biológicas para poder hacer predicciones y modelamientos a nivel molecular, determinar interacciones entre proteínas, llevando a entregar buenos aportes en el área de la farmacéutica para la formulación de medicamentos y vacunas (125,126).

Desde los inicios de la investigación del VLB en los años 80s se ha planteado la posibilidad de la transmisión al humano y ha generado la inquietud de su potencial zoonótico, a pesar que para la

época los resultados no fueron concluyentes debido a las herramientas tecnológicas disponibles en su momento (20,36). A medida que la ciencia evoluciona, se dispone de nuevas tecnologías y herramientas que han permitido mejorar las técnicas moleculares, celulares e inmunológicas enfocadas al diagnóstico y seguimiento a nivel de laboratorio de estos patógenos, lo que ha permitido para el caso del VLB avanzar en el conocimiento de la infección de este virus en el humano y en otras especies (1,44,45).

El VLB se ha propuesto como un agente versátil debido a su capacidad de infección a especies distintas al bovino tanto en condiciones naturales como experimentales (19,20,127). A nivel de cultivo celular se ha demostrado la infección en células distintas a su célula blanco (linfocitos B de bovinos) como células epiteliales mamarias de bovino (128), células dendríticas (23) e incluso células provenientes de otras especies animales como monos, conejos, cerdos, gatos, ratones y ovejas (24,25). Para los virus la interacción de sus proteínas más externas con los receptores celulares define el tropismo viral. Para el caso de los virus que infectan múltiples especies se plantea que pueden haber receptores compartidos entre especies con regiones conservadas que permiten la unión a las proteínas virales y de esta manera se puedan infectar tanto los hospederos naturales como los accidentales (129).

Para el caso del VLB se han descrito proteínas de transporte como la AP3D1 (29) y el complejo de transporte de aminoácidos catiónicos CAT1/SLC7A1 (30) como potenciales receptores celulares que interactúan con la proteína gp51 del virus para mediar el ingreso a la célula. Estas proteínas de transporte son comunes entre los mamíferos con altos porcentajes de identidad entre especies, lo que podría explicar el ingreso del virus a múltiples hospederos (130,131).

Como parte del trabajo colaborativo en el laboratorio de virología realizamos un modelamiento *in silico* de la proteína AP3D1 de bovino para identificar las interacciones puntuales con la proteína gp51 del virus, y se realizó un análisis comparativo del AP3D1 bovino (boAP3D1) con el del humano, en el cual se identificaron regiones conservadas entre el AP3D1 de estas dos especies, incluyendo los principales sitios de unión con la proteína gp51 lo que sugeriría el ingreso del virus a las células humanas (Anexo 3). Las interacciones entre boAP3D1 y gp51 se verificaron con

ensayos *in vitro* de las proteínas recombinantes, confirmando los resultados obtenidos con la aproximación *in silico.*

Con respecto a las aproximaciones experimentales en los humanos, existe poca evidencia de la capacidad de infección del virus en células humanas, así como del impacto de la infección (26,28,132). Con el fin de aportar evidencia experimental de la capacidad de infección del virus en los humanos, se planteó un estudio de infección de distintas líneas celulares de origen humano para determinar si a nivel de laboratorio el virus lograba ingresar y establecerse en células humanas provenientes de diferentes órganos, como soporte al planteamiento del potencial zoonótico del virus (Artículo 6). Con este estudio se investigó la susceptibilidad de infección de 9 líneas celulares humanas a la infección del VLB bajo condiciones controladas utilizando la estrategia de infección de co-cultivo con las células FLK (provenientes de riñón fetal de cordero y persistentemente infectadas con el VLB), buscando lograr la infección en las células humanas. Se realizó un seguimiento por 6 meses a los cultivos con el fin de identificar si alguna de las líneas celulares humanas infectadas con el virus lograba mantener una infección estable la cual se pudiera seguir utilizando como un modelo a nivel de laboratorio para futuros estudios. De las líneas celulares evaluadas se logró la infección del virus en 7 de las 9 líneas celulares, de las cuales las líneas iSLK proveniente de riñón y MCF7 proveniente de tejido mamario demostraron una infección estable en el tiempo.

Con los resultados obtenidos en esta sección hemos aportado al conocimiento del potencial zoonótico del VLB a nivel experimental. Si bien aún es necesario realizar más estudios para entender en su totalidad el impacto que tiene el VLB en el humano y su patogenicidad, con los resultados presentados en esta tesis doctoral se abordaron varios de los principios de las infecciones zoonóticas incluyendo (68,93): prevalencias actualizadas del virus en el hospedero natural (el bovino), circulación del virus en el humano con frecuencias similares a los bovinos, asociación con una enfermedad en el humano (factor de riesgo para el cáncer de mama), identificación de hospederos accidentales en el entorno natural como los búfalos y las ovejas, identificación de potenciales vías de diseminación hacia el humano a través de la presencia del virus en alimentos de consumo, identificación de marcadores moleculares compartidos entre especies con distribuciones heterogéneas en los árboles filogenéticos indicando que no existen

tipos virales específicos de especies animales, así como la presencia de haplotipos virales compartidos entre humanos y bovinos, y finalmente, evidencia experimental de la capacidad de infección del virus en líneas celulares de origen humano y del receptor celular.

Todos estos resultados aportan evidencia contundente del potencial zoonótico del virus de la leucosis bovina y que es una infección que puede estarse transmitiendo desde los bovinos a los humanos, atravesando las barreras de especie, lo que nos lleva a buscar alternativas para la implementación de medidas adecuadas de prevención y control que involucren tanto la salud animal como la humana, así como medidas de seguridad alimentaria para disminuir las cadenas de contagio y ojalá en un futuro, poder plantear programas de erradicación del virus como ya ocurre en algunos países de Europa.

Artículo 6: Olaya-Galán, N.N.; Blume, S.; Tong, K.; Shen, H.; Buehring, G.C. In vitro susceptibility of infection of human cell lines to the bovine leukemia virus (Accepted). Frontiers in Microbiology. 2021, XX, 1–14.

In vitro susceptibility of human cell lines infection by bovine leukemia virus

Nury N. Olaya-Galán[1,2*], Skyler Blume[3], Kan Tong[3], HuaMin Shen[3], Maria F. Gutierrez[2], Gertrude C. Buehring[3*]

[1]PhD Program in Biomedical and Biological Sciences, School of Medicine and Human Health, Universidad del Rosario, Bogotá, Colombia

[2]Grupo de Enfermedades Infecciosas, Laboratorio de Virología, Departamento de Microbiología, Pontificia Universidad Javeriana, Bogotá, Colombia

[3]School of Public Health, University of California, Berkeley, CA, USA

*** Correspondence:**
Nury N. Olaya-Galán
nury.olaya@urosario.edu.co

Gertrude C. Buehring
buehring@berkeley.edu

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Abstract

Evidence of the presence of bovine leukemia virus (BLV) in human beings and its association with breast cancer has been published in the literature, proposing it as a zoonotic infection. However, not enough evidence exists about transmission pathways nor biological mechanisms in human beings. This study was aimed at gathering experimental evidence about susceptibility of human cell lines to BLV infection. Malignant and non-malignant human cell lines were co-cultured with BLV-infected FLK cells using a cell-to-cell model of infection. Infected human cell lines were harvested and cultured for three to six months to determine stability of infection. BLV detection was performed through liquid phase PCR and visualized through in situ PCR. Seven out of nine cell lines were susceptible to BLV infection as determined by at least one positive liquid-PCR result in the three-month culture period. iSLK and MCF7 cell lines were able to produce a stable infection throughout the three-month period, with both cytoplasmic and/or nuclear BLV-DNA visualized by IS-PCR. Our results support experimental evidence of BLV infection in humans by demonstrating the susceptibility of human cells to BLV infection, supporting the hypothesis of a natural transmission from cattle to humans.

1 Introduction

Viral agents have been linked to approximately 20% of human cancer types, and some causative relationships have been established (1). The most common examples of those relationships include human papilloma virus (HPV) with cervical cancer, hepatitis B and C viruses (HBV, HCV) with liver cancer, Epstein-Barr virus (EBV) with Burkitt's lymphoma and human herpes virus 8 (HHV8) with Kaposi's sarcoma. Breast cancer has long been studied as a possible candidate for a virus-caused human cancer, due to the evidence of some viral markers present on breast cancer tissues (2–4). In the last decade, bovine leukemia virus (BLV) has been proposed as a possible risk factor for breast cancer development in different regions as a result of case-control studies in which statistically significant associations of the presence of the virus with breast cancer patients has been identified (5–9).

BLV is an exogenous retrovirus, grouped with human T cell leukemia virus (HTLV) in the deltaretrovirus genus. These viruses cause leukemia/lymphoma both in cattle and humans respectively (10). Cancer development could take more than 5-10 years post-infection to occur, although a low percentage (about 5-10%) of the infected population develop the last stages of the disease. BLV is distributed worldwide with prevalence rates between 10-90% in cattle, although North and South America have some of the highest prevalence rates (70-90%) (11). One of the biggest challenges of BLV infection, is that most of the infected animals remain asymptomatic in the herds favoring the transmission and dissemination processes as no vaccine is available and diagnosis is not performed broadly (12).

Previous research has reported the presence of BLV biomarkers in humans, such as gene segments, viral proteins, and antibodies against BLV, which provides clear evidence of the presence of the virus in this host (13–19). Studies of BLV in humans have been based on epidemiological analyses for the viral detection and association with breast cancer, but still there is a gap in the knowledge, viz. how does this virus (which naturally infects cattle) reach the human population and infect human beings. Therefore, concerns about the zoonotic potential of BLV have been present for some time in the BLV research, and for several years, researchers have tried to show the implications of BLV in human beings (15,20,21). In early BLV research it was not possible to identify any relationship between humans and BLV (20), but now there is increasing evidence about the presence of the virus in human beings, strengthening the hypothesis of BLV being a zoonotic agent (22–25).

Even if cattle is the natural host of the virus, evidence of the presence of BLV has also been reported in other species (26–28). Thus, BLV has been described as a versatile agent which could infect multiple hosts both naturally and under controlled conditions in the laboratory (29). In cattle, the target cells of the virus are the B lymphocytes (30), although in other studies *in vitro* infection has been evaluated in cell lines of different origins/sources including other bovine tissues, and some other animal species (30–34). However, few studies have been carried out regarding the infection of BLV in human cell lines and its implications of infection (35–37).

For BLV and its close relative HTLV, low amounts of free viral particles are released in the viral cycles compared with other retroviruses, and thus, a cell-to-cell transmission is needed to reach uninfected cells (10,38). Although it is rare and less efficient, free viral particles could also be released from infected cells and perform a classic viral cycle of infection mediated by cellular receptors such as AP3D1 or CAT1/SLC7A1 proteins (39,40). This research was focused on investigating if human cell lines from different tissues were susceptible of infection with the BLV under controlled conditions in the laboratory. Our results provide evidence supporting the hypothesis

of the zoonotic potential of the virus and providing a model of human infection for further studies, as stable infection was reached in two different human cell lines.

2 Materials and Methods

2.1 Cell lines and culture conditions

Fetal Lamb Kidney (FLK) cells, constitutively infected with BLV, served as a repository of the virus. Minimal Essential Medium (MEM) was used for cell passage every 3-4 days after 80% confluency. Human cell lines used and growing conditions are described in the table below (Table 1) (41–49). For each experiment, viability was verified by trypan blue stain and cells were counted through Neubauer's chamber technique.

Table 1.

2.2 TransWell Infections

In order to recreate a plausible scenario of infection of BLV, TransWells (Costar Corning Inc.) with 0.4 μm polyester pore membrane, were inserted above a 12 well plate to co-culture BLV infected cells with human cell lines. The TransWell pores do not allow whole cells to pass through the pores but do allow extensions of cells (e.g., nanotubes or cellular conduits) to make contact below the insert, allowing a cell-to-cell transmission of the virus. Before infections, cell lines were verified to be negative to BLV (Supplementary figure 1).

Uninfected human cell lines were cultured in the lower compartments with an approximate concentration of 10^5 cells/mL until 70-80% confluency was reached in their respective media (Table 1). Thereafter, FLK cells were seeded in the TransWells (6000 cells per well). For experimental infections co-cultures were incubated for 48 hours and then, the human cell lines in the lower compartment were harvested. The BLV-infected human cell lines were scaled up into T25 cell culture flasks for follow-up of the infection, for up to three - six months to determine the stability of BLV infection.

As experimental controls, human cell lines with PBS 1X instead of FLK cells in the TranWell were also collected and maintained simultaneously with infected ones in order to compare morphology or any other visible change caused due to the viral infection. Infection experiments were repeated twice for the study, at two independent and different times. Manipulation of cell lines was carried out independently, reducing the risk of cross-contamination of cell cultures. Infections per cell line were performed on different days, and for the maintenance and follow-up, separate hoods for infected and non-infected cell lines were used as well. In addition, cell lines were initially validated to be negative to BLV prior infection with the PCRs used for viral detection (see below).

2.3 BLV detection and follow-up of infection

After 48 hours post infection (p.i.) with the TransWells, an aliquot of the cells in the bottom plate was recovered to perform DNA extraction with the DNeasy Kit from QIAgen following the manufacturer's instructions. Total DNA recovered was stored at -20ºC until further use. Human GAPDH housekeeping gene was used as a validation control of the DNA extraction. Sheep *cytochrome C oxidase* housekeeping gene was used to verify that FLK cells did not trespass the membrane of the TransWells and human cell lines were not contaminated with FLK.

Viral genome was detected by PCR (see below). Successful infected human cell lines were considered those in which BLV-DNA was detected in the initial DNA extraction post-infection and were maintained in cell culture with its respective conditions (Table 1). A follow up of the positive cell lines was performed every two weeks in which an aliquot of infected cells and controls without infection were taken for DNA extraction and PCR detection.

Viral genes *LTR, gag, pol, env* and *tax* were tested by liquid-phase nested PCR (nPCR) with GoTaq Promega in order to verify the presence of the complete genome of the virus at time 0 post infection. Primers used for the viral detection and PCR conditions were used from previous studies (16,39). For the follow-up, detection of the GRE region of the LTR of the virus was used as a bioamarker of viral infection, as it is one of the most conserved genes of the virus and belong to the 5' limit of the viral genome. Results were visualized by electrophoresis in an 1.5% agarose gel, TBE1x, stained with ethidium bromide, and run conditions for 30 min – 100V in TBE1x. PCR conditions and primers are shown in the supplementary table 1.

For those cell lines which showed a stable infection of BLV during the total time of the follow-up, an *in situ* PCR (IS-PCR) was performed after 16 weeks of infection to confirm the presence of the virus inside the cells using methodology adapted from Nuovo (50). Cell cultures were detached, rinsed, and smeared on enhanced adherence glass microscope slides (SuperFrost – Fisher ®). The slides were air dried and fixed for 18 hours in 10% formalin neutral buffer. Digestion was performed with 2 mg/mL pepsin in 0.1 N HCl (20 min), followed by pepsin inactivation solution (100 mmol/L Tris-HCl, 100 mmol/L NaCl, pH 7.4) applied for 1 min, and were rinsed in DPBS and a final wash in absolute ethanol. Samples, run in duplicate, were surrounded with a 15 × 15 mm frame seal chamber (Bio-Rad, Hercules, CA, USA) for the PCR mix.

The PCR mixture was 4.0 mmol/L MgCl2, 0.4 mmol/L dNTPs, 1 µmol/L primers (Operon Biotechnologies, Huntsville, AL, USA), 0.06% bovine serum albumin, 8 µmol/L digoxigenin-11-dUTP (dig) (Hoffman-La Roche, Basel, Switzerland), and 0.053 U/µL of HotStart Amplitaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA). IS-PCR was directed to a segment of the *tax* region of the BLV genome (nt 7197-7570, F: CTTCGGGATCCATTACCTGA and R: GCTCGAAGGGGGAAAGTGAA), with an expected product of 373 bp. PCR mix was placed into the chambers of the slides and was sealed with the plastic cover of BioRad. Slides were placed into an IS-PCR machine (Hybaid Thermo OmniSlide; Cambridge Biosystems, Cambridge, UK) for amplification. Thermal profile was used as previously described (16). After amplification, endogenous peroxidase was quenched 30 min in 3% hydrogen-peroxide solution prepared in methanol. Dig-labeled nucleotides incorporated into PCR products were detected by anti-dig antibodies in an avidin-biotin-immunoperoxidase reaction (Hoffman-La Roche) and were revealed by diaminobenzidine (DAB) solution followed by the manufacturer's instruction (Vector ®, Burlingame – CA, USA VECTOR ®). Smears of FLK cell line were used as a positive control. As a negative control of reaction, an adjacent smear for each cell line prepared without Taq polymerase and without primers, was evaluated to verify that no cross-reaction or non-specific attachment occurred by the DIG-labelled uracil and/or by the anti-dig monoclonal antibody. Results were visualized in a Nikon Eclipse E200 optical microscope under 40x magnification. Visualization of dark brown-red stain was considered a positive result. After verification of stable infection after 16 weeks, cells were stored in liquid nitrogen for further analyses.

3 Results

All of the aliquots obtained from cell culture were validated for human GAPDH after 48h.p.i. and in each aliquot after DNA extraction for the follow-up. *Cytochrome C oxidase* of sheep was negative in all of them, confirming that it was not a cross-contamination from FLK (Supplementary figures 2 and 3).

Success of TransWell infection and stability of infection varied among different cell lines. CaSki and G361 cell lines were negative for most BLV genes following incubation in TransWells. DLDI, 293T, Raji, MCF-102A, HS-27, MCF-7 and iSLK cell lines were susceptible to BLV infection, as detected by nPCR amplification of viral genes. In time zero post infection, all of the amplified regions for viral detection were present in 7 out of the 9 cell lines (Figure 1).

Figure 1.

The DLDI, 293T, Raji, MCF-102A and HS-27 cells were unable to sustain infection. In these cells, detection of BLV DNA decreased with time and became nondetectable over the following up period. The results of nested and *in situ* PCR are represented on figures 2 and 3 respectively, in which cell lines that reached a stable infection are described.

The presence of the GRE biomarker in the cell lines throughout the three month follow up (figure 2) is evidence of a stable infection. MCF-7 and iSLK were positive during the complete follow-up. No discernable changes in morphology of the cells infected with BLV were noted.

Figure 3 shows the results of *in situ* PCR as an end-point experiment performed on MCF-7 and iSLK after 16 weeks post-infection to visually validate the results of nPCR on cell lines with stable infection. Areas of the cells stained red/dark brown indicate regions containing BLV DNA. The cell lines iSLK and MCF7 showed BLV-*tax* gene segment inside the cells evenly distributed, with some darker spots visualized in the nucleus of iSLK (Fig. 3 – 100x).

After 18 months after freezing in liquid nitrogen, cells were recovered again in cell culture to verify if still were infected. An immunohistochemistry directed to p24 protein of the virus was carried out and detection was performed with DAB reagent in an immunoperoxidase system. Presence of the virus was confirmed in MCF-7 cells as visualized with dark coloring inside the cells. In addition, multinucleated cells were visualized suggesting the presence of syncytia formation (Supplementary figure 4). iSLK cells were not possible to recover after thawing.

Table 2.

Figure 2.

Figure 3.

4 Discussion

Understanding the impact that BLV infection could have in human beings is a topic of concern. Previous evidence of BLV in humans has reported its presence in blood, lung, and breast tissues, from people with and without cancer (13,16,18,19). This study shows that several human cell lines from different tissues were susceptible to BLV infection, and that a stable infection was obtained in two of the evaluated cell lines (MCF-7 and iSLK). Results showed the presence of viral DNA which could be as a provirus, as total DNA was extracted for the follow-ups. Susceptibility of infection was shown in cells from different tissues such as kidney, colon, fibroblasts, and breast. These results open the possibility that BLV could be present in other organs in human beings, and considering its oncogenic potential, it will be interesting to evaluate if there is an association with other cancer etiologies in addition to breast (23) and lung cancer (51).

Previous evidence of BLV *in vitro* infections has been focused on understanding specific pathways and molecular mechanisms of the virus (30,32–34,52–54). However, few studies included human cell lines in the experimental designs (35,37,54). Results published by Altaner et. al. were directed to evaluate the implications of BLV infection in humans' cells of neurotropic origin (35). Establishing an *in vitro* model that will allow the research community for further studies in the biological mechanisms of BLV infection in humans is still a priority in the research field. We propose that iSLK and MCF-7 cell lines could be used in the future for further analyses.

Previously, Suzuki et. al. analyzed the early stages of the *in vitro* infection of the virus, in which it was determined that one of the crucial stages for viral producing cell lines and for stable infections was the retrotransciption (37). As a retrovirus, once BLV enters the host cell its RNA genome is expected to be retrotranscribed to viral DNA and remains as extrachromosomal double strained DNA (E-DNA). Unlike retroviruses such as HIV, translocation of the viral genome into the nucleus is weak and mainly relies on cell division processes (55). However, once into the nucleus, integration in the host genome occurs mediated by viral integrases in which a proviral state is acquired, generating a stable and persistent infections in the host (56,57). When E-DNA remains in the cytoplasm of the cell and does not managed to be translocated into the nucleus, viral DNA is more likely to degrade inside the cells and is not possible to obtain stable infections (58).

Our results showed that seven out of the nine cell lines were able to overpass the initial steps of the viral cycle, such as attachment, entrance and retrotranscription. Even if it was expected a cell-to-cell infection as few amounts of free viral particles are released, independently of which was the mechanism used by the virus for the entrance in human cell lines, evidence of DNA obtained from the extractions in the follow-up indicated that initial steps were successfully fulfilled. Viral receptors proposed for BLV (AP3D1 and CAT1/SLC7A1) (39,40) are proteins which are widely distributed in most of the eukaryotic cells, as are proteins involved in intracellular transport processes. Those proteins could be favoring the interactions between the virus and different host cells due to the high identity and similarity percentages among species (59,60), and their low specificity with tissues and specific cells. For the case of CaSki and G361 cell lines, which were the only two cell lines in which the infection was not successful, it might be due to lower amounts of expression of the receptor proteins compared with other cell types (61,62) or to the lack of capacity of inducing nanotubes formation for the viral entrance (38,63).

The cell lines in which BLV genome was detected for a longer term supports the hypothesis of a stable infection in MCF-7 (breast), iSLK (kidney) and DLD-I (colon) cell lines. Even if in our study we did not measure active viral production, nor functionality of the viral genes, the identification of

these cell lines with the presence of viral DNA after a long-term of infection suggest that a stable infection in the human body may occur as well. Although the present study does not suggest a mechanism as to why certain cell lines were unable to maintain a stable BLV infection, we hypothesis that it could be related with the process of integration in the host genome. Considering that BLV does not have a specific profile for integration within the host genome, and that it occurs randomly in different regions, with different patterns and in several chromosomes in cattle without a specific association with disease progression (64,65), this also could be occurring in the human cells. In cattle, it is expected to integrate about 2 to 6 copies of BLV genome in the host cells, what favors to the increase of proviral load in the host due to the clonal expansion related to the disease (29). As there is no correlation for the integration profiles in which different number of copies could be integrated, in our experiments, changes in the intensity of the bands present in the electrophoresis and darker colors in the *in situ* PCR could be associated with higher amounts of viral genome. The localization of BLV in the nucleus in iSLK vs the cytoplasm for MCF-7 based on the results from IS-PCR could be interpreted as differences in the integration profiles of the BLV in the form of provirus. It could be associated with more copies integrated in iSLK in contrast with MCF-7, however, in this study we did not look for the integration sites of the virus, as it was not the main objective of the manuscript. Finally, stable and persistent infections could also be related with the cell cycles of the different cell lines, in which initial cells that were infected with BLV could die before its mitotic cycle or could be displaced by uninfected cells replacing the cells populations.

One of the strengths of this study were the steps taken to ensure that the results obtained were free from cross-contamination. All DNA samples extracted from the human cell cultures were negative in PCR amplification of sheep cytochrome C which makes it unlikely that positive nested PCR results were due to FLK cell-line contamination. Furthermore, FLK control DNA and cell line were handled in different areas, separate from the human cell culture samples to avoid cross-contamination while processing and to ensure the positive results obtained from DNA within the human cell culture samples. Two different detection techniques were used as well to ensure reliability of our results. IS-PCR visualization showed presence of the viral genome inside the cells, which could be both in the cytoplasm of the cells or in the nucleus. Even if liquid-phase PCR is unable to provide any information as to the location of the source of the DNA, it showed the presence of viral genome after a total DNA extraction, indicating that early steps of the viral cycle were performed.

In addition, more than one BLV genome region was used for the follow up of the experiments. The LTR (long terminal repeat) promoter region of BLV was used for liquid phase-PCR, and *tax* region which codes an auxiliary protein with oncogenic potential was used for IS-PCR. Those regions were chosen because are the most highly conserved regions of BLV genome (26,66). It is thought that in BLV and HTLV, the gag- pol (polymerase) - env segment of the BLV genome is often deleted during the progression of the disease to escape the host's immune response (29,67). Detection of genomic biomarkers as evidence of the viral infection by PCR could result in false negatives if these regions were the primary or sole target for assays. Furthermore, it is important to highlight that both LTR region and Tax protein are crucial for active transcription of the virus within the host cells, as Tax protein acts as the main transactivator of the LTR promoter region, inducing active transcription of the virus (68,69). In further studies it will be interesting to determine levels of expression of both biomarkers and to identify if is there any correlation with the cell cycle, viral transcription and cellular transformation patterns.

A limitation of our current study is that the positive results from the liquid phase-PCR do not provide information as to the integration status of BLV with respect to the host genome. While these results

show that viral retro-transcription occurred (33), we could not be sure if the virus was able to integrate its genome with that of the host cell, which is an essential step for its replication and persistent infection. This is an area of future research as inverse PCR techniques allow for determination of the integration status of the retrovirus, and can also provide information about where in the host's genome the virus integrates, which could give an insight of its localization in the host genome and of possible cellular genes' alteration (70). However, inverse PCR assays are challenging as large amounts of PCR products are required for sequencing, that could be influenced by the proviral load in the host. Further studies are needed to understand the impact of the infection of BLV in these cell lines and in human beings.

Of special interest, the mammary epithelial cell line MCF-7 was both susceptible to BLV infection and able to maintain a stable infection over the three-month culture period. Both conditions seem to be important in the progression of the disease in its host (10). This result is significant as it provides *in vitro* experimental evidence consistent with the hypothesis of BLV as an exogenous etiological agent of human breast cancer, obtained through a zoonotic infection (24). Nevertheless, the varying susceptibility and capacity of maintaining a stable infection of BLV in human cell lines as well as from other mammal species, opens new questions in BLV research in which further studies are needed towards the characterization of the mechanisms involved in BLV infection in other hosts than cattle. Our results provide evidence of BLV being capable of infecting human cell lines, what supports the hypothesis of natural infection of BLV in human beings, with huge possibilities of infecting different tissues among the human body.

5 Conclusions

BLV was able to infect human cell lines through a cell-to-cell model. BLV biomarkers were identified in the cell lines as evidence of a stable infection. iSLK and MCF-7 cell lines were those which showed positive for a longer term, even after freezing and thawing. Our results support the hypothesis of BLV being a zoonotic infection and are the basis for further studies for a better understanding of BLV mechanisms in human beings. Stable infected cell lines will be useful for future studies to be performed under controlled conditions at the laboratory level.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

Conceptualization, NOG and GCB; methodology, NOG, KT, SB, HMS; software, NOG; validation, GB; formal analysis, NOG, SB, HMS, GCB; investigation, NOG, MFG, GCB; resources, GCB; writing—original draft preparation, NOG, SB; writing—review and editing, NOG, MFG, GCB; visualization, NOG, GCB; supervision, MFG, GCB; project administration, GCB; funding acquisition, GCB. All authors have read and agreed to the published version of the manuscript.

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11 Data Availability Statement

All data was supplied within the text.

12 Figures captions

Figure 1. BLV genes' fragments for infected human cell lines at time 0 post-infection. 1.5% agarose electrophoresis gels with representative results per cell line. Results are shown at 48 h.p.i of the TransWell system. Each infection of the cell lines was repeated twice in two independent moments. DNA from FLK cell line was used as a positive control for PCR reactions.

Figure 2. Three-month follow-up of BLV infection in human cell lines with GRE region. Gel electrophoresis of PCR product (285 bp) run on 1.5% agarose TBE gel, stained with ethidium bromide. Lanes in order are of DNA extracted two weeks, one month, two months, and three months after TransWell infection. Notice high intensity of the bands on iSLK and MCF-7 cell lines. In 293T cell line, viral infection was lost after week 4 post-infection.

Figure 3. Evidence of BLV inside the cells through *in situ* PCR (*tax* region). Smear of infected cell lines. FLK – positive control cell line. iSLK-BLV positive (kidney). MCF-7 BLV positive (breast). Antibody control – *in situ* PCR without primers and TaqPolymerase control, verification of no cross-reaction of anti-dig antibody. *In situ* PCR with complete reaction. Stain is visualized by DAB reaction against anti-dig system. Images visualized at 40x and 100x on a Nikon Eclipse E200 optical microscope. All images were treated with the same conditions of color and contrast.

13 Tables

Table 1. Cell lines used in *in vitro* infection experiments and growth conditions.

CELL LINE	Cell/Tissue type	Biological status	Medium + 10% FBS	Passage (days)	Ref
RaJi (ATCC CCL-86)	B cell - Lymphoblast	Burkitt's lymphoma + EBV	RPMI-1640	2-3	42
HS-27 (ATCC CRL-1634)	Fibroblast - Skin	Non-malignant	DMEM	7-8	43
MCF 102A (ATCC CRL-10781)	Epithelial - Mammary gland	Non-malignant	DMEM/F12, 1:1	3-4	44
MCF 7 (ATCC HTB-22)	Epithelial - Mammary gland	Adenocarinoma	DMEM	4-5	45
CaSki (ATCC CRL-1550)	Epithelial - Cervix	Epidermoid carcinoma + HPV	RPMI-1640	3-4	46
G361 (ATCC CRL-1424)	Epithelial - Skin	Malignant melanoma	MEM	3-4	47
293T (ATCC CRL-3216)	Epithelial - Embryonic kidney	Non-malignant + Adenovirus	DMEM	2-3	48
DLD-I (ATCC CCL-221)	Epithelial - Colon	Colorectal carcinoma	RPMI-1640	4-5	49
iSLK (UC Berkeley donation)	Epithelial - Kidney	Renal carcinoma + latent KSHV	DMEM	4-5	50

567 **Table 2.** Evidence of BLV-GRE genomic region in human cell lines after infection through
568 nPCR in a follow-up of 12 weeks post infection.

CELL LINE	REPLICATE	EVIDENCE OF BLV POST-INFECTION				
		t0	week 2	week 4	week 8	week 12
RaJi (ATCC CCL-86)	1	Pos	Pos	Neg	Neg	Neg
	2	Pos	Pos	Neg	Neg	Neg
HS-27 (ATCC CRL-1634)	1	Pos	Pos	Pos	Pos	Neg
	2	Pos	Pos	Pos	Neg	Neg
MCF 102A (ATCC CRL-10781)	1	Pos	Pos	Pos	Neg	Neg
	2	Pos	Pos	Pos	Neg	Neg
MCF 7 (ATCC HTB-22)	1	Pos	Pos	Pos	Pos	Pos
	2	Pos	Pos	Pos	Neg	Neg
CaSki (ATCC CRL-1550)	1	Neg	-	-	-	-
	2	Neg	-	-	-	-
G361 (ATCC CRL-1424)	1	Neg	-	-	-	-
	2	Neg	-	-	-	-
293T (ATCC CRL-3216)	1	Pos	Neg	Neg	Neg	Neg
	2	Pos	Pos	Neg	Neg	Neg
DLD-I (ATCC CCL-221)	1	Pos	Pos	Pos	Neg	Neg
	2	Pos	Pos	Pos	Neg	Neg
iSLK	1	Pos	Pos	Pos	Pos	Pos
	2	Pos	Pos	Pos	Pos	Pos

569

Ladder 100 bp
FLK
Neg (H2O)
MCF10
Raji
HS27
MCF7
CaSki
G361
293T
ISLK
DLDI
307 bp
LTR

Ladder 100 bp
FLK
Neg (H2O)
MCF10
Raji
HS27
MCF7
CaSki
G361
293T
ISLK
DLDI
272 bp
gag

Ladder 100 bp
FLK
Neg (H2O)
MCF10
Raji
HS27
MCF7
CaSki
G361
293T
ISLK
DLDI
150 bp
pol

Ladder 100 bp
FLK
MCF10
Raji
Neg (H2O)
MCF7
CaSki
G361
293T
ISLK
DLDI
HS27
701 bp
env

Ladder 100 bp
FLK
Neg (H2O)
MCF10
Raji
HS27
MCF7
CaSki
G361
293T
ISLK
DLDI
373 bp
tax

Fig 1.

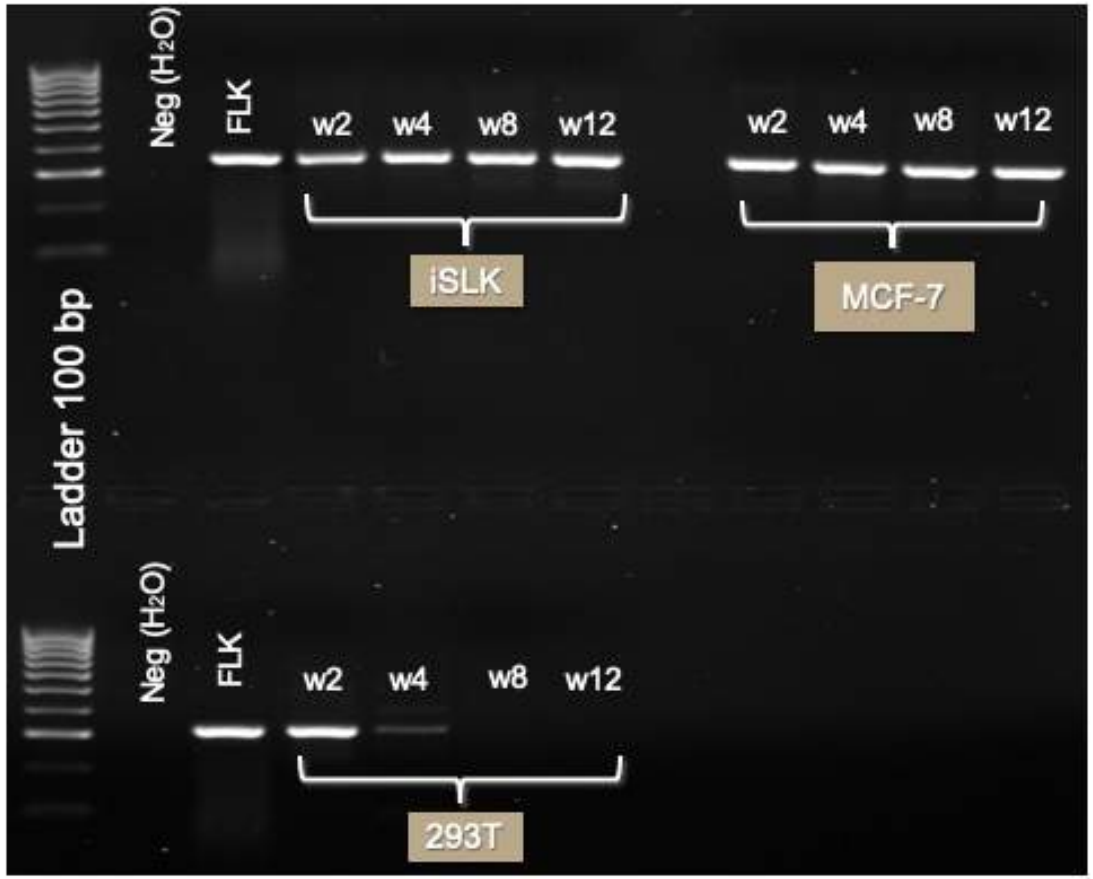
Ladder 100 bp
Neg (H2O)
FLK
w2
w4
w8
w12
iSLK
w2
w4
w8
w12
MCF-7
Neg (H2O)
FLK
w2
w4
w8
w12
293T

Fig. 2.

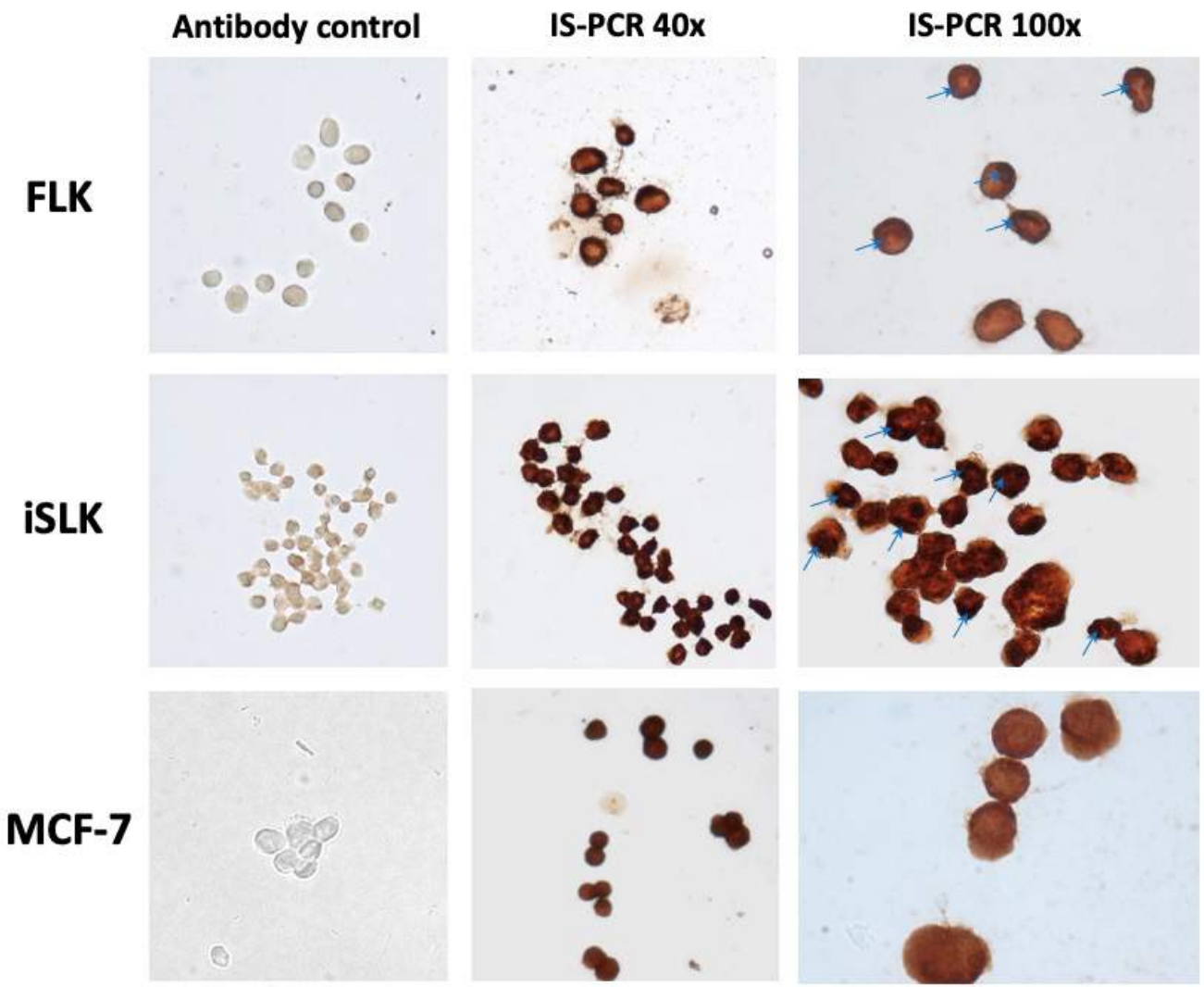

Fig. 3.

8. CONCLUSIONES

1. La presencia del VLB en la población evaluada aumenta el riesgo del desarrollo de cáncer de mama dado al valor de OR encontrado para el estudio.

2. La presencia de segmentos génicos del VLB en productos alimenticios sugiere que las carnes y leches como una vía de salida del bovino.

3. Existen haplotipos virales compartidos y evidencias de recombinación entre bovinos, humanos y alimentos planteando potenciales vías de transmisión.

4. La presencia del virus en hospederos accidentales aumenta el riesgo de diseminación y transmisión.

5. Según la encuesta epidemiológica realizada, el consumo de leche de ordeño y derivados lácteos pueden aumentar el riesgo de adquisición del virus en la población evaluada.

6. Se propone al VLB como una potencial infección zoonótica proveniente de los bovinos.

Conclusión global:

Los resultados obtenidos en esta investigación aportan evidencias del potencial zoonótico del VLB como una infección proveniente de los bovinos hacia los humanos dando respuesta a los principios de las infecciones zoonóticas: presencia del virus en su hospedero natural (bovinos) y hospederos accidentales (humanos, ovejas y búfalos) con altos porcentajes de identidad de sus marcadores moleculares entre especies, identificación de potenciales vías de diseminación a través de alimentos provenientes de bovinos infectados, evidencia experimental bajo condiciones controladas de la capacidad de infección del virus al humano y la identificación del receptor con el cual interactúan las proteínas virales de superficie.

9. PERSPECTIVAS Y RECOMENDACIONES

Si bien, el conjunto de los resultados obtenidos en este estudio aporta a la investigación actual del VLB y su relación con los humanos, muchas son las preguntas que quedan por resolver para futuras investigaciones. Los estudios epidemiológicos enfocados a la búsqueda de nuevos factores de riesgo que puedan estar asociados con enfermedades como el cáncer son la base de las evidencias de causalidad que deben ser replicados en diferentes regiones geográficas. Los resultados obtenidos en esta investigación concuerdan con evidencias previas registradas para el VLB en otros países donde plantean al virus como un factor de riesgo para el desarrollo de la enfermedad.

La evidencia experimental de la capacidad de infección del virus en células humanas es la base para poder caracterizar esta infección en el humano como hospedero accidental, en lo posible enfocado a estudios que demuestren su capacidad de transformación. Las líneas celulares infectadas fueron almacenadas y se espera desarrollar más análisis de éstas al interior del grupo de investigación, como sus potenciales sitios de inserción en el genoma celular, y la capacidad de infección de células humanas a otras células. Así mismo, se propone realizar infecciones dirigidas a células linfocitarias humanas, teniendo en cuenta la célula blanco del virus. Dentro del grupo de investigación se plantea la posibilidad de realizar otros estudios de casos y controles con otros tipos de cáncer en los cuales el virus pueda estar involucrado, tomando como base las líneas celulares de colon y de riñón que fueron susceptibles de infección por el virus y que además demostraron la capacidad de mantener una infección estable.

Si bien estos resultados soportan la hipótesis de una infección zoonótica proveniente de los bovinos, aún queda una última pregunta sobre la transmisión del virus al humano, y es el punto de unión entre la vía de diseminación, y el ingreso efectivo del virus al humano a través del curso natural de la infección. Para esto, es necesario verificar la liberación ya sea de partículas virales infectivas, o el contacto con células infectadas que garantice el paso del virus al humano. Se propone realizar ensayos *in vivo* en modelos animales utilizando como fuente de diseminación del virus productos cárnicos y lácteos positivos para el virus, para determinar si a través de estos se logra una infección exitosa en los modelos animales.

Por último, se propone generar estrategias de prevención y control en el ganado bovino, así como en otros animales criados en granjas para el manejo de la infección del virus en su entorno natural. Bajo el principio de "Una Salud", donde se propone analizar las situaciones de salud de una manera global, incluyendo el bienestar animal, humano y ambiental considerado como un todo y como un ciclo que se conecta entre sí, una buena alternativa es plantear procesos de erradicación del virus como ocurrió en algunos países de Europa, para disminuir las tasas de prevalencia del VLB en los animales, garantizando una mejor calidad de vida para los mismos, reducción de las pérdidas económicas ocasionadas en el gremio ganadero y a futuro, evitar que este virus llegue al humano, al ser considerado un potencial factor de riesgo para el desarrollo del cáncer.

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