UNIVERSIDAD DE LA REPÚBLICA

MONTEVIDEO-URUGUAY

PROGRAMA DE DESARROLLO DE LAS CIENCIAS BÁSICAS

PEDECIBA

CARACTERIZACIÓN MOLECULAR Y ANÁLISIS DE VARIABILIDAD GENÉTICA DE LOS VIRUS RESPIRATORIO SINCITIAL Y METAPNEUMOVIRUS HUMANO AISLADOS EN PANAMÁ, DURANTE LOS AÑOS 2008-2012

Por

LEYDA E. ÁBREGO SÁNCHEZ

Orientador: Dr. Juan Arbiza

Co-orientadores: Dra. Adriana Delfraro y Dr. Juan Pascale

Tesis Sometida a Consideración del Tribunal Examinador del Programa de Desarrollo de Las Ciencias Básicas para Optar al Grado de Doctorado

2017

TABLA DE CONTENIDO

	Pág
Agradecimientos	2
RESUMEN	3.
INTRODUCIÓN	4
1. Importancia	4
2. Antecedentes	4
3. Taxonomía	7
4. Estructura y Genoma	8
4.1 VRSH	8
4.2 MPVH	12
5. Replicación Viral	14
5.1 VRSH	14
5.2 MPVH	16
6. Estacionalidad de VRSH y MPVH	18
7. Epidemiología Molecular	19
7.1 VRSH	19
7.2 MPVH	22
8. Vacunas	23
8.1 VRSH	23
8.2 MPVH	24
JUSTIFICACIÓN	25
OBJETIVOS	26
HIPÓTESIS	25
CAPITULO I	28
Caracterización genética y antigénica de cepas del VRSH grupo A, aisladas en Panamá entre 2008-2012.	
CAPITULO II	54
Caracterización genética de cepas del VRSH grupo B, aisladas en Panamá entre 2008-2012.	
CAPITULO III	65
Caracterización molecular de cepas de Metapneumovirus Humano aislados en Panamá, durante los años 2008-2012.	
CONCLUSIONES Y PRESPECTIVAS	81
MATERIAL SUPLEMENTARIO	84
REFERENCIAS BIBLIOGRAFICAS	86

AGRADECIMIENTO

A Dios por la vida, guiarme, darme siempre las fuerzas para seguir adelante y abrirme las puertas para prepararme profesionalmente y logar mis metas.

A mi padre y a mi madre, por todo el esfuerzo que hicieron para brindarme un futuro lleno de esperanzas y mejores oportunidades. Y por seguir siempre estando presente en mi vida para apoyarme en lo que necesitara y cuidar de mis hijos.

A mis hermanas por estar siempre a mi lado, apoyándome en cada una de mis decisiones profesionales. A mi prima, Ingrith Alaín Sánchez, por creer en mí, por su apoyo incondicional y anhelo de verme terminar.

A Zeuz por ser mi compañero de batalla, brindándome siempre todo el apoyo que estuviera a su alcance. Por sus palabras de aliento y valiosos consejos. Y a mis hijos por tratar de entenderme y portarse bien.

Agradezco muy sinceramente a mis tutores: Al Dr. Juan Ramón Arbiza, por haber visto un potencial en mí y ofrecerme la oportunidad de seguir creciendo profesionalmente, para que tuviera mejores oportunidades y poder dar luces de un futuro mejor para mis hijos. Al Dr. Juan Miguel Pascale, por creer en mí sin conocerme y ofrecerme siempre todo su apoyo. A la Dra. Adriana Delfraro, por adoptarme, por bríndame una mano amiga, por entenderme, por su paciencia y estar siempre pronta a darme una salida sencilla y encontrar una solución rápida y certera; y no permitir que me estancara sin antes haber hecho el intento.

Un sincero agradecimiento a la Universidad de la República, Montevideo. Por aceptarme dentro de su programa PEDECIBA y hoy, hacer una realidad mi crecimiento profesional, cuando me sentí limitada a no poder realizar un Doctorado.

Al Instituto Carlos III en Madrid, España. En especial al Dr. José Antonio Melero y la Dra. Alfonsina Trento, por todo su apoyo y enseñanzas. Por permitirme realizar mi pasantía y publicar con ustedes.

Al Instituto Conmemorativo Gorgas de estudios de la Salud “ICGES”, por abrirme las puertas cuando no encontraba dónde trabajar. En especial al Dr. Néstor Sosa. Al Departamento de Investigación en Virología y Biotecnología, por acogerme con alegría y brindarme todo el apoyo económico, facilidades de los laboratorios y equipos necesarios para la realización de esta tesis. A mi Jefa, Brechla Morenos y todos mis compañeros del Departamento; en especial a Marlene Castillo, María Cano, Melissa Gaitán, Danilo Franco, Sandra López Vergès, Yaneth Pittí, José Valenzuela y Ruth Helleby; por apoyarme siempre, compartir mis alegrías y brindarme palabras de aliento en momentos de tristezas y desesperación.

A la Secretaría Nacional de Ciencias, Tecnología e Innovación Panamá (SENACYT); Grant número: CAP10-001 (JA) y Al Sistema Nacional de Investigación (SIN). Agencia Nacional de Investigación e Innovación y Programa de Desarrollo de las Ciencias Básicas del Uruguay.

RESUMEN

En este trabajo de investigación se realizó la caracterización molecular y análisis de variabilidad genética de los virus respiratorio sincitial humano (VRSH) y metapneumovirus humano (MPVH) aislados en Panamá, durante los años 2008-2012. Además, también se probaron anticuerpos monoclonales (MAb) contra la proteína G, de cepas representativas de varios genotipos del virus respiratorio sincitial del grupo A (VRSH-A), aisladas en diferentes años (1990-93 y 2012). También se realizó un análisis evolutivo, utilizando secuencias del gen G reportadas a nivel mundial. Los resultados obtenidos de ambos análisis genéticos y antigénicos para el VRSH-A, demostraron que a pesar de la alta variabilidad genética que se reporta para la proteína G de VRSH, antigénicamente esta proteína parece estar conservada, al menos en los epítopos estudiados. En cuanto al análisis filogenético realizado para las cepas panameñas del virus respiratorio sincitial del grupo B (VHSH-B), todas las secuencias compartieron la característica de la duplicación de 60 nucleótidos descrita para el genotipo BA. Seis cepas panameñas agruparon con el genotipo BA10 y 12 muestras agruparon en un clado monofilético separado con un valor de soporte de 0,92 y una distancia genética (p-distance) intragrupo inferior a 0,07; cumpliendo con los criterios para ser considerado un nuevo genotipo, al que denominamos BA14. Las otras seis cepas no mostraron una agrupación clara, pero parecen estar estrechamente relacionadas con los genotipos BA9, BA11, o el nuevo BA14, de acuerdo al grado de homología nucleotídica que presentan. Diferentes sustituciones de aminoácidos fueron observadas en las cepas de VRSH-B panameñas, algunas anteriormente descritas y otras encontradas sólo en cepas de Panamá. La caracterización molecular de MPVH, mediante el análisis del gen N, mostró la co-circulación de cuatro sub-linajes: A2a, A2b, B1 y B2. El linaje A1 no fue observado en las cepas panameñas analizadas en este estudio. El sub-linaje A2b fue el más frecuente. La mayoría de las cepas panameñas de los sub-linajes A2a y A2b fueron muestras recolectadas en la epidemia de 2012. Las cepas del sub-linaje B1, a su vez, se obtuvieron en 2010, mientras que las muestras pertenecientes a la sub-línea B2 fueron recolectadas durante todo el período del estudio (2010-2012).

INTRODUCCIÓN

1. Importancia

Las infecciones agudas del tracto respiratorio bajo, son una de las principales causas de morbilidad y mortalidad en niños a nivel mundial. La Organización Mundial de la Salud, ha clasificado estas infecciones; como la segunda más importante causa de muerte en niños menores de cinco años (Bryce et al., 2005; Black et al., 2010). *Streptococcus pneumoniae*, *Haemophilus influenzae* y el virus respiratorio sincitial humano (VRSH), son los principales patógenos asociados con la neumonía infantil (Rudan et al., 2008). Estudios basados en etiología viral han identificado al VRSH en un 15-40% de los casos de neumonía o bronquiolitis en niños, seguido por Parainfluenza virus (PIVs) Influenza virus , MPVH y Adenovirus (Rudan et al., 2008). A pesar de que la frecuencia de las infecciones respiratorias producidas por MPVH (5-15%) no son tan altas en comparación con el VRSH, la sintomatología que provoca al infectar las vías respiratorias bajas, es severa en pacientes en riesgo e indistinguible de la producida por VRSH , es aquí donde radica la importancia de estudiar de ambos virus.

2. Antecedentes

En 1956, Morris et al; describieron un agente viral que producía un efecto citopático característico en cultivos celulares y que provocaba una enfermedad respiratoria en chimpancés caracterizada por tos, estornudos y secreción mucopurolenta. Este patógeno fue denominado agente de la coriza en chimpancés (ACC) Posteriormente, Chanock y Finberg, 1957, informaron sobre dos aislados de lactantes con enfermedad respiratoria grave

(bronconeumonía, bronquiolitis y laringo-traqueo-bronquitis). Estos dos virus humanos fueron indistinguibles del ACC descrito por Morris et al., 1956; y propusieron llamarlos, virus respiratorio sincitial. Tres años más tarde, Beem et al., 1960, describieron este virus con un efecto citopático inusual sobre las células HEp-2, caracterizado por la formación de células gigantes multinucleadas, al que llamaron virus Randal. Luego realizaron pruebas de neutralización cruzada utilizando sueros hiperinmunes de conejo obtenidos a partir de las cepas de virus de chimpancé (Sue), de lactante (Long) y Randal, demostrando similitud antigénica entre los tres virus. En la actualidad, el VRSH no sólo es una causa importante de infección respiratoria grave en niños menores de 5 años, sino también en ancianos y pacientes inmunocomprometidos e incluso en adultos previamente sanos (Dowell et al., 1996, Hashem and Hall, 2003). Además de la morbilidad aguda causada por el VRSH, numerosos estudios desde hace más de 40 años han descrito la asociación entre la infección por VRSH en la infancia y el desarrollo posterior de sibilancias recurrentes y asma durante la primera década de vida (Stein et al.,1999; Henderson et al., 2005; Sigurs et al., 2005; Escobar et al., 2010; Wu and Hartert, 2011).

El MPVH fue detectado por primera vez en la línea celular de mono Rhesus (LLC-MK2), inoculada con muestras colectadas de niños con infección respiratoria de posible etiología viral, donde el agente no pudo ser identificado. En estos cultivos celulares se observó un efecto citopático indistinguible de los inducidos por el VRSH. El sobrenadante de células infectadas analizado por microscopía electrónica, reveló partículas pleomórficas midiendo de 150 a 600 nm, con proyecciones cortas de 13 a 17 nm. Este sobrenadante no mostró actividad hemoaglutinante, y la propagación del virus se observó que era dependiente de la tripsina (van den Hoogen et al., 2001). El análisis de secuencias genómicas reveló un

genoma viral relacionado con metapneumovirus aviar (MPVA). El MPVA ha sido clasificado en cuatro subgrupos, que van del A al D (Bäyon-Auboyer et al., 2000), dentro de los cuales MPVA-C está más relacionado con MPVH. Sin embargo, la identidad en cuanto a la secuencia de las proteínas de unión (G) e hidrofóbica pequeña (SH) entre MPVA y MPVH es solamente de un 20-30%, mientras que el porcentaje de identidad para las otras proteínas estructurales es de 80% (van den Hoogen et al., 2002).

Los síntomas del MPVH son diversos, pero todos asociados al tracto respiratorio. En los niños infectados con MPVH se observan síntomas como rinorrea, fiebre y tos; con menos frecuencia presentan vómito, diarrea y conjuntivitis (van den Hoogen et al., 2004). Las enfermedades asociadas al MPVH más frecuentes son bronquiolitis, neumonía, tos seca y exacerbación del asma. Muchas veces los síntomas son similares a los de otras infecciones respiratorias virales comunes y no se hace un diagnóstico clínico certero. A pesar de lo anterior, ciertas diferencias clínicas pueden ocurrir y ayudar al diagnóstico, por ejemplo, la fiebre ≥39°C es más común en infecciones con MPVH, mientras que la rinorrea es más común en pacientes con VRSH. (Hara et al., 2008; Beneriet al., 2009).

El periodo de incubación del virus, es entre 4 – 6 días y su excreción se extiende por un período de 2 semanas (van den Hoogen et al., 2003; Ebihara et al., 2004; Williams, et al., 2004; Feuillet et al., 2012). Sólo un estudio ha encontrado ARN de MPVH en suero utilizando RT-PCR (Maggi et al., 2003), lo cual fortalece el hecho que este virus se encuentra limitado al tracto respiratorio. Estudios realizados en roedores y primates no humanos no han detectado al MPVH en otros tejidos que no pertenecen al tracto respiratorio (Kuiken et al., 2004; Alvarez et al., 2004; Hamelin et al., 2005; Williams et al., 2005; Wyde et al., 2005).

La población en riesgo son principalmente niños, pacientes inmunocomprometidos y adultos mayores (Boivin et al., 2003; van den Hoogen et al., 2003, van den Hoogen, 2007). Las personas inmunosuprimidas pueden ver su salud seriamente comprometida si son infectados por MPVH, ya que es capaz de causar severas infecciones. Esto no sólo ha sido descrito para este virus, sino para otros virus respiratorios. Existen reportes de pacientes que fallecieron por MPVH con cáncer y muchos estudios sugieren serios problemas por este virus en pacientes que han recibido trasplante de células madre (Pelletier et al., 2002; Cane et al., 2003; Boeckh et al., 2005; Franquet et al., 2005; Williams et al., 2005). El MPVH en los adultos mayores probablemente esté sub-reportado ya que no se hacen análisis para este virus en los adultos mayores hospitalizados. Dentro de este grupo, las poblaciones que están más expuestas son las que habitan en asilos o pacientes hospitalizados (Fearns and Collins, 1999; Williams et al., 2005).

3. Taxonomía

El VRSH y el MPVH pertenecían a la sub familia *Pneumovirinae*, pero recientemente, según el Comité Internacional de Taxonomía de Virus (siglas en inglés: ICTV), la subfamilia *Pneumovirinae*, fue establecida como una familia "*Pneumoviridae*". El género *Pneumovirus* fue renombrado "*Orthopneumovirus*", para evitar confusión entre la familia y los miembros del género. Por lo que el VRSH y el MPVH se encuentran reclasificados de la siguiente forma (Afonso et al., 2016):

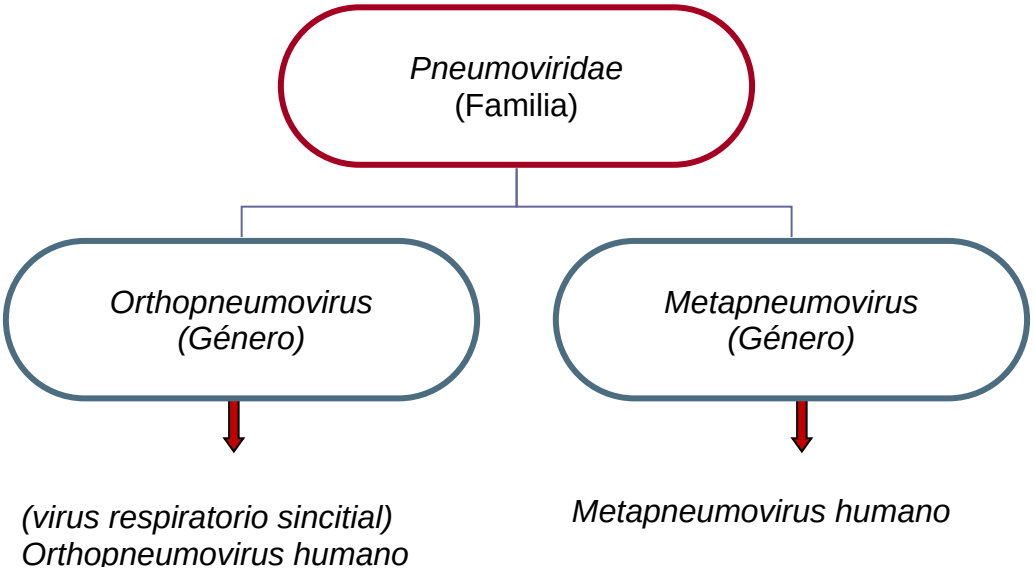


Figura 1. Esquema de clasificación taxonómica de los virus respiratorio sincitial y metapneumovirus humano.

4. Estructura y Genoma

4.1. VRSH

Estructuralmente, el virión de VRSH consiste en una nucleocápside empaquetada en una envoltura lipídica que deriva de la membrana plasmática de la célula hospedera. Su genoma es ARN de hebra simple y polaridad negativa, no segmentado; el cual se encuentra cubierto en su totalidad por la proteína N para formar una nucleocápside estable que lo protege de degradación. El tamaño del genoma tiene un rango de 15.192 a 15.226 nucleótidos (Collins et al., 2013). El virión producido en cultivo celular consiste de una partícula esférica de 100 a 350 nm en diámetro encontrándose además partículas filamentosas que usualmente predominan y son de 60-200 nm en diámetro pudiendo alcanzar los 10 µm de largo. El genoma se compone de 10 genes virales que son transcriptos secuencialmente por separado en ARNm por la polimerasa viral. Estos genes están ordenados linealmente desde el extremo 3' al extremo 5' del ARN genómico. En el extremo 3' están los genes NS1, NS2 (proteínas no estructurales 1 y 2, con actividad antiinflamatorias), N (nucleoproteína) y P

(fosfoproteína, que actúa como cofactor en la síntesis de ARN). Estos están seguidos por M (proteína de la matriz), SH (pequeña proteína hidrófoba, una proteína no esencial que puede funcionar como un canal iónico pentamérico, G (glicoproteína de membrana integral tipo II, cuya función es la adsorción a la célula huésped), y F (proteína integral de membrana tipo I, responsable de la fusión entre la envoltura viral y la membrana celular). La mayor parte de la región 5' corresponde a M2 (un gen que abarca dos marcos de lectura abiertos que codifican M2-1 y M2-2, relacionadas a la transcripción del ARNm y balance entre la transcripción y replicación del ARN) y L (ARN polimerasa viral) (Figs. 1 y 2) (Hall, 2001; Collins and Graham, 2008; Hurwitz, 2011; Collins et al., 2013).

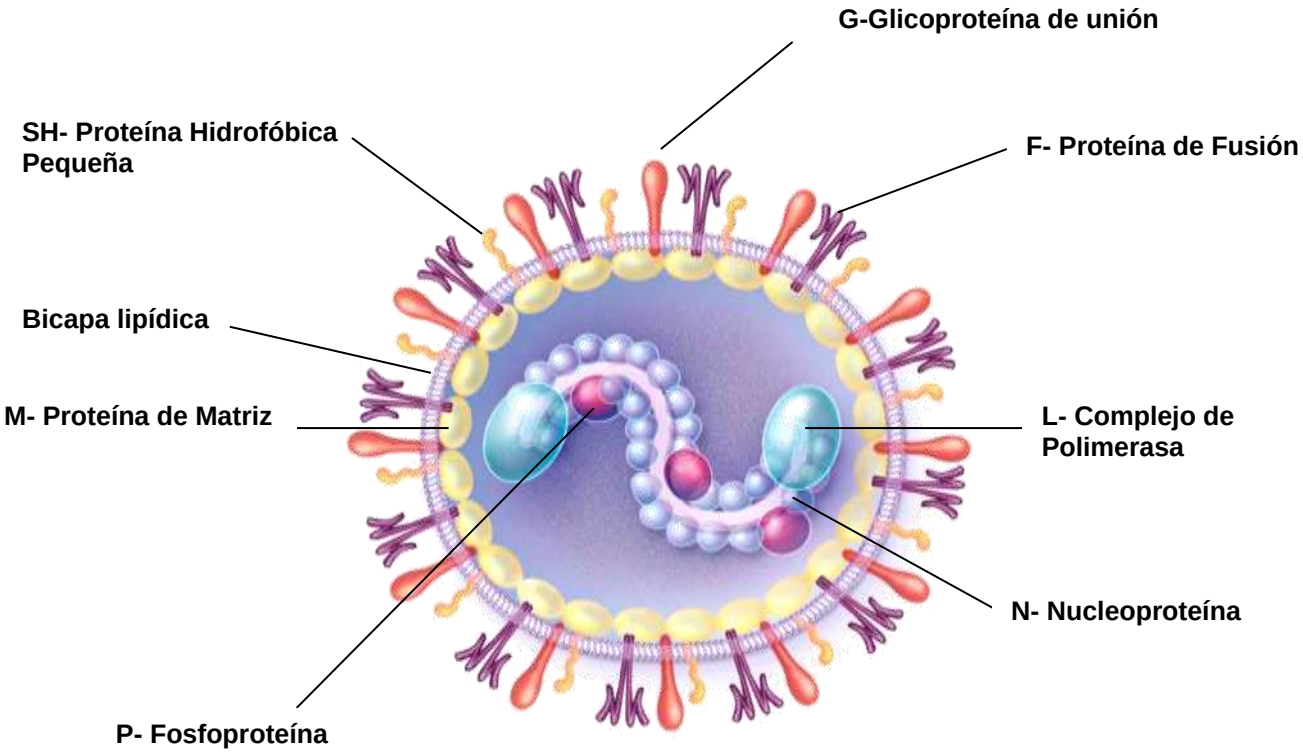


Figura 2. Estructura del VRSH. (Extraído y traducido de Hall,2001).

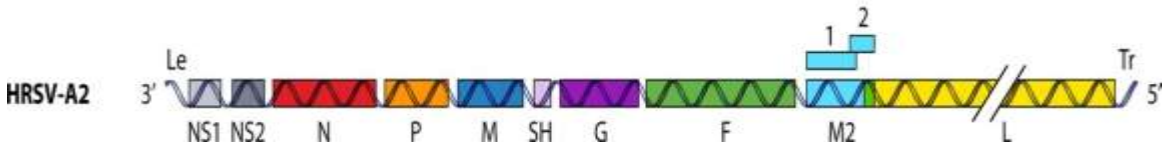


Figura 3. Mapa genómico del VRSH. Cada fragmento coloreado indica el gen abreviado, N nucleoproteína, P, fosfoproteína, M, proteína de la matriz; F, la proteína de fusión, SH, proteína hidrofóbica pequeña, G, la proteína de unión, L, proteína polimerasa grande, Tr, remolque, NS1 y NS2, proteínas no estructurales 1 y 2. (Extraído de Schildgen et al., 2011).

La proteína G varía de 282-321 aminoácidos de largo y se encuentra fuertemente glicosilada. Presenta al menos 30 sitios potenciales de N y O glicosilación, los cuales en su mayoría se encuentran pobremente conservados (Johnson et al., 1987). (Wathen et al., 1991). En su estructura se han determinado 5 sitios putativos de N-glicosilación y al menos 25 sitios potenciales de O-glicosilación, incrementando su peso molecular de 32 KDa a 90 KDa (Wathen et al, 1991, Hurwitz, 2011, Collins and Graham, 2008; Collins et al., 2007, Cui et al., 2013). Esta proteína (Fig.4) presenta tres regiones: la cola citoplasmática (AAs 1-38), el dominio transmembrana (AAs 38-66), y el ectodominio (AAs 66-298). El ectodominio C-terminal de la proteína G se compone de 2 dos dominios hipervariables altamente glicosilados (HVR1 y HVR2), que flanquean el sitio de unión del receptor putativo, con una región conservada de 13 aminoácidos (AAs 164-176) situada entre ellos. (Wertz et al., 1985, Johnson et al., 1987; Roberts et al., 1994; Zlateva et al., 2005). Las dos regiones variables del ectodominio contienen altas concentraciones de residuos de serina y treonina, los cuales son potenciales sitios aceptores de O glicosilación. Las uniones tipo N y O de oligosacáridos contribuyen a la estructura antigénica de la proteína G (Lambert 1988; García et al., 1996, Palomo et al., 2000). La proteína G de VRSH también existe como una proteína secretada, producto de un codón de iniciación alternativo en la región de

codificación del dominio transmembrana (Graham, 2011; Hurwitz, 2011; Robets et al.,1994). Algunos investigadores sugieren que el producto secretado puede actuar como un señuelo para bloquear la respuesta de anticuerpos neutralizantes G-específicos del hospedero (Graham, 2011; Hurwitz, 2011; Bukreyev et al., 2008).

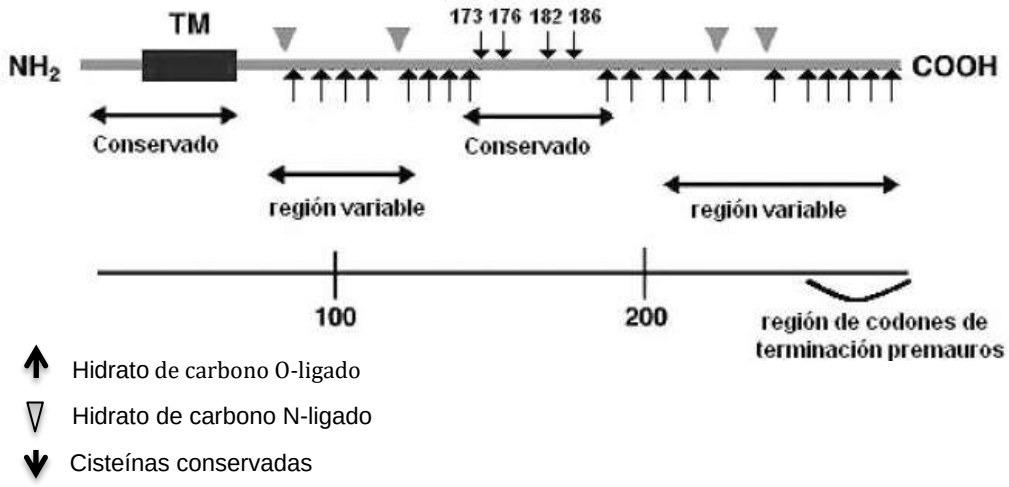


Figura 4. Estructura primaria de la glicoproteína G del VRSH. Esta figura muestra las regiones variables y conservadas de la proteína G. Los sitios O y N- glucosilados. Los sitios O-glucosilados se indican con una flecha hacia arriba y los N- con un triángulo gris invertido. Se indican también las posiciones de las 4 cisteínas conservadas y la región de codones de terminación prematura. (imagen extraída de Viegas et al., 2011).

La proteína F consta aproximadamente de 500 aminoácidos y es mucho menos glicosilada que la proteína G, produciendo una glicoproteína de 70-kDa. Esta proteína se sintetiza en primer lugar como una forma F_0 precursora inactiva que se ensambla en un homotrímero (Collins et al ., 2007; Hurwitz, 2011). El precursor es sensible a la ruptura por una proteasa intracelular tipo furina en el trans-Golgi de la célula infectada, aunque la ruptura completa no se requiere para el transporte de F a la superficie celular (Hurwitz, 2011; Sugrue et al., 2001; Dutch et al., 2001). Se obtienen tres productos de esta ruptura: F2, p27 y F1. La F1 contiene el dominio transmembrana en un extremo, dos repeticiones heptadas (HRA y HRB), y un péptido de fusión hidrofóbico en el extremo opuesto. La formación de un

enlace disulfuro entre las subunidades F1-F2 es esencial, esta forma es la fusogénicamente activa y es indispensable para la fusión virus/célula o la fusión células/célula, la cual promueve la formación de sincitios (Russell and Luque, 2006). La SH es la tercera proteína integral de membrana. Esta proteína consta de 64 aminoácidos y está anclada cerca del extremo N, con el extremo C orientado extracelularmente. La mayor parte de la proteína SH no se encuentra glicosilada (Carter et al., 2010; Gan et al., 2012). La proteína SH, parece ser una viroporina, una clase de pequeñas proteínas virales que pueden modificar la permeabilidad de la membrana y pueden afectar el proceso de brotamiento y la apoptosis celular; además parece inhibir la señalización de TNF-α, una citoquina antiviral (Fuentes et al., 2007). El VRSH recombinante que carece de SH puede reproducirse algo más eficientemente in vitro, presumiblemente debido a su menor tamaño genómico.(Whitehead et al., 1999, Collins et al., 2013).

4.2. MPVH

Similar a otros pneumovirus, el virión de MPVH contiene una envoltura de membrana lipídica rodeando la matriz (M) y tres glicoproteínas transmembrana de superficie: F, G, y SH. Dentro de la envoltura se encuentra una ribonucleoproteína helicoidal compleja (RNP), la cual consiste de nucleoproteína (N), fosfoproteína (F), polimerasa (L), y el ARN genómico, consistente en una hebra simple de ARN no segmentada de polaridad negativa (Fig. 5).

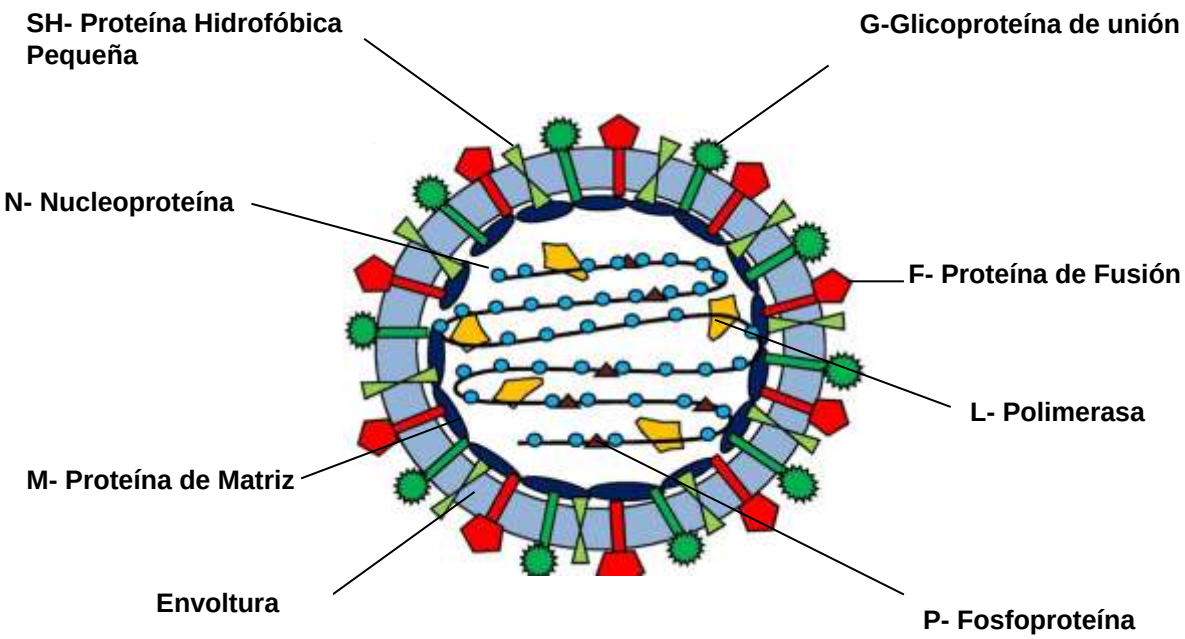


Figura 5. Estructura del metapneumovirus humano. Extraído y traducido de Feuillet *et al*., 2012.

El genoma tiene un rango de tamaño de 13.280 a 13.378 nucleótidos y contiene ocho genes que codifican para 9 proteínas. Similar al VRSH, el genoma del MPVH posee el gen M2, el cual consta de dos marcos de lectura (ORF) a partir de los cuales se expresan las proteínas M2-1 y M2-2. Sin embargo, a diferencia de VRSH, MPVH carece de genes no estructurales (NS1 y NS2), y difiere además en el orden de los genes (Fig. 5 y 6). (van den Hoogen et al., 2001 y 2002; Schildgen et al., 2011; Feuillet et al., 2012).

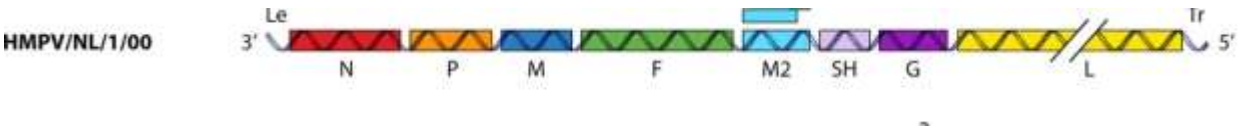


Figura 6. Mapa genómico de MPVH. Cada fragmento coloreado indica el gen abreviado. N nucleoproteína, P, fosfoproteína, M, proteína de la matriz; F, la proteína de fusión, SH, proteína hidrofóbica pequeña, G, la proteína de unión, L, proteína polimerasa grande. Extraído de Schildgen et al ., 2011.

5. Replicación Viral

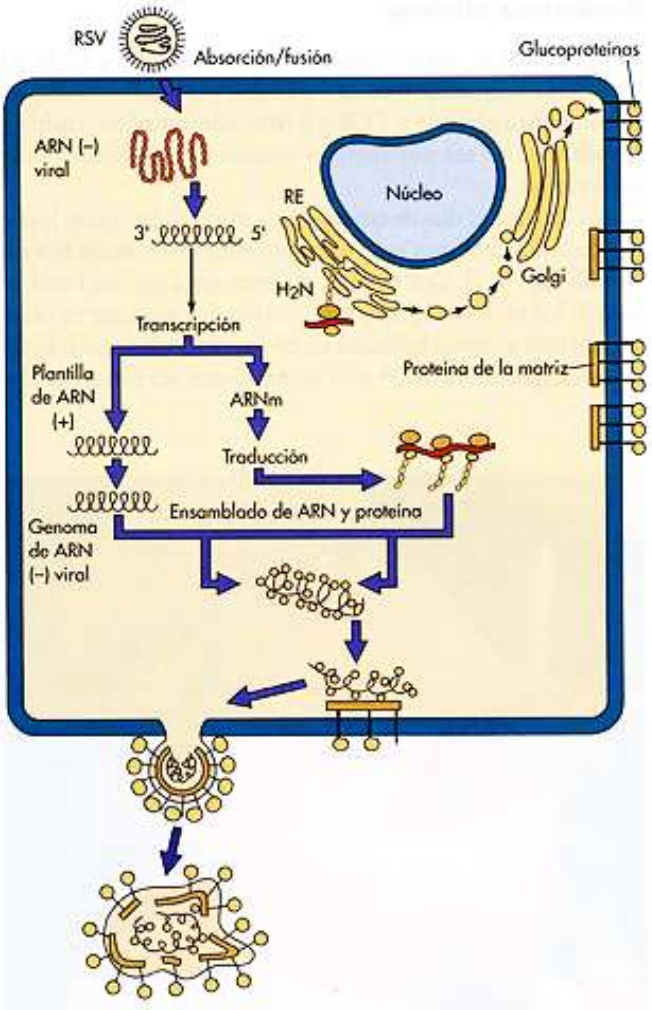
5.1. VRSH

El ciclo de vida del virus comienza por la unión de la glicoproteína G a la membrana plasmática de la célula del hospedero mamífero (F también puede facilitar la unión), seguido por fusión mediada por la proteína F a nivel de la superficie celular. Las células diana de las proteínas F y G no están totalmente identificadas. La proteína G puede unir glucosaminoglicanos tales como el heparán sulfato y condroitín sulfato B, como también lectinas tipo-C tales como la proteína surfactante (Graham, 2011; Levine et al., 2001). La proteína F al igual que la G, puede unir glucosaminoglicanos (Feldman et al., 2000, Techaarpornkul et al., 2002; Hallak et al., 2000). Otros receptores posibles para VRSH incluyen la molécula de adhesión intercelular-1 y el receptor de quimiocinas CX3CR1(Collins and Graham, 2008; Collins et al., 2007; Tripp, 2001).

La fusión virus-célula se produce cuando el péptido de fusión se inserta en la membrana de la célula diana y la proteína se repliega en una estructura de horquilla asistida por interacciones entre las secuencias heptadas repetidas HRA y HRB para atraer las membranas celular y viral en estrecha proximidad (Russell and Luque, 2006). Los virus que carecen de la glicoproteína G (Delta G) son capaces de fusionar e infectar *in vitro*, aunque los virus Delta G son altamente atenuados *in vivo* (Collins et al., 2007). Una vez que las membranas del virus y la célula se fusionan, los componentes internos virales se liberan en el citoplasma, donde se produce la replicación del HRSV. Las proteínas P y L participan en la síntesis de ARN, tanto para la producción de ARNm como para la producción de nuevos genomas de cadena negativa (~ 15 kb de tamaño). Durante la transcripción y la replicación, la nucleoproteína protege el genoma y anti-genoma de ARN. Tal blindaje del ARN por la nucleoproteína ayuda a evitar el inicio de los mecanismos de

defensa del huésped por TLR y otras formas de señalización. Seguido del nuevo ARN y la producción de proteínas, los nuevos viriones se ensamblan en la cercanía de la superficie de la célula, donde adquieren la envoltura, siendo liberados de la célula por brotamiento o gemación (Fig.7) (Collins and Graham, 2008; Collins et al., 2007).

Figura 7. Ciclo de replicación del VRSH. El ciclo inicia con la unión de la proteína G del VRSH a la



membrana de la célula hospedera, seguida por la fusión mediada por la proteína F. Una vez dentro, los componentes internos virales se liberan en el citoplasma, y se produce la síntesis de ARN genómico y ARNm. Seguido del nuevo ARN y la producción de proteínas, los nuevos viriones se ensamblan en la cercanía de la superficie de la célula, donde adquieren la envoltura, y son liberados de la célula por gemación (imagen de Murray et al., 2009).

5.2. MPVH

El ciclo de replicación del MPVH inicia con la unión del virus a la célula hospedadora, la cual está dirigida por la proteína G (Lamb y Parks, 2007). La proteína G es la proteína más variable de los MPVH (van Woensel et al., 2006). La secuencia de aminoácidos de la proteína G contiene una región simple hidrofóbica que está localizada cerca del amino N-terminal y se cree que sirve como una señal peptídica de anclaje a la membrana. Cerca de tres cuartos de la región carboxilo terminal, corresponde al dominio extracelular. La proteína G de este virus, tiene un alto contenido de serina y treonina, los cuales son sitios potenciales aceptores para O - glicosilación, y además posee un alto contenido de prolina (van den Hoogen et al., 2002) característica compartida con estructuras tipo mucina altamente glicosiladas. La estructura deducida de aminoácidos de la proteína G se confirmó mediante análisis de la biosíntesis, glicosilación, transporte intracelular y expresión de la proteína G en la superficie de la célula (Liu y Bastien, 2007). Se ha sugerido que los glicosaminoglicanos incluyen moléculas de sulfato de heparina que están relacionadas con la unión de la proteína G a la célula hospedadora (Thammawat et al., 2008). Virus recombinantes sin la proteína G son capaces de replicarse tanto *in vitro* como *in vivo*, indicando que la unión vía proteína G no es un paso indispensable en el ciclo de replicación (Biacchesi et al., 2004 y 2005).

La fusión de la membrana viral con la membrana de la célula hospedadora es mediada por la proteína F. La organización estructural de esta proteína es similar a las proteínas de fusión de clase I de otros virus, en donde la proteína F es sintetizada como un precursor F0 que requiere el corte por proteasas para activar las subunidades F1 y F2, las cuales se mantienen unidas por puentes disulfuro (Lamb y Jardetzky, 2007). La fusión de

membranas promovida por las proteínas F de la familia *Pneumoviridae* generalmente ocurre a nivel de la membrana plasmática de la célula hospedadora a un pH neutro (Lamb, 1993; Russell y Luque, 2006.). Sin embargo, varios estudios han demostrado la fusión celular durante la infección por cepas del grupo A del MPVH, a pH ácido. Son necesarios estudios adicionales para caracterizar el mecanismo preciso de fusión en estos virus (Schowalter et al., 2009; Herfst et al., 2008).

Una vez que la nucleocápside viral está en el interior del citoplasma, las proteínas P, N y L se disocian del ARN viral y a su vez se unen formando el complejo de polimerasa. El ARN genómico puede ser utilizado entonces como molde para la transcripción y replicación víricas, la cual ocurre enteramente en el citoplasma de las células infectadas. Las proteínas recién producidas P, N, L y M2 se asocian con los genomas virales sintetizados "de novo" para formar nuevas nucleocápsides que se incorporarán a los viriones durante el proceso de gemación en la superficie celular. Las glicoproteínas de la envoltura (F, G, SH) hacen su camino a través del aparato de Golgi a estas zonas de acumulación membranosa y asociadas a ellas, para finalmente ser expuestas en la superficie de la célula infectada. También se ha observado la progresión de la infección en cultivo celular a través de la formación de sincitios. Por este mecanismo, la célula infectada se fusiona con las células adyacentes a través de la acción de la proteína F expuesta en su superficie, permitiendo la propagación célula-célula del virus (Fig.8) (Feuillet et al., 2012).

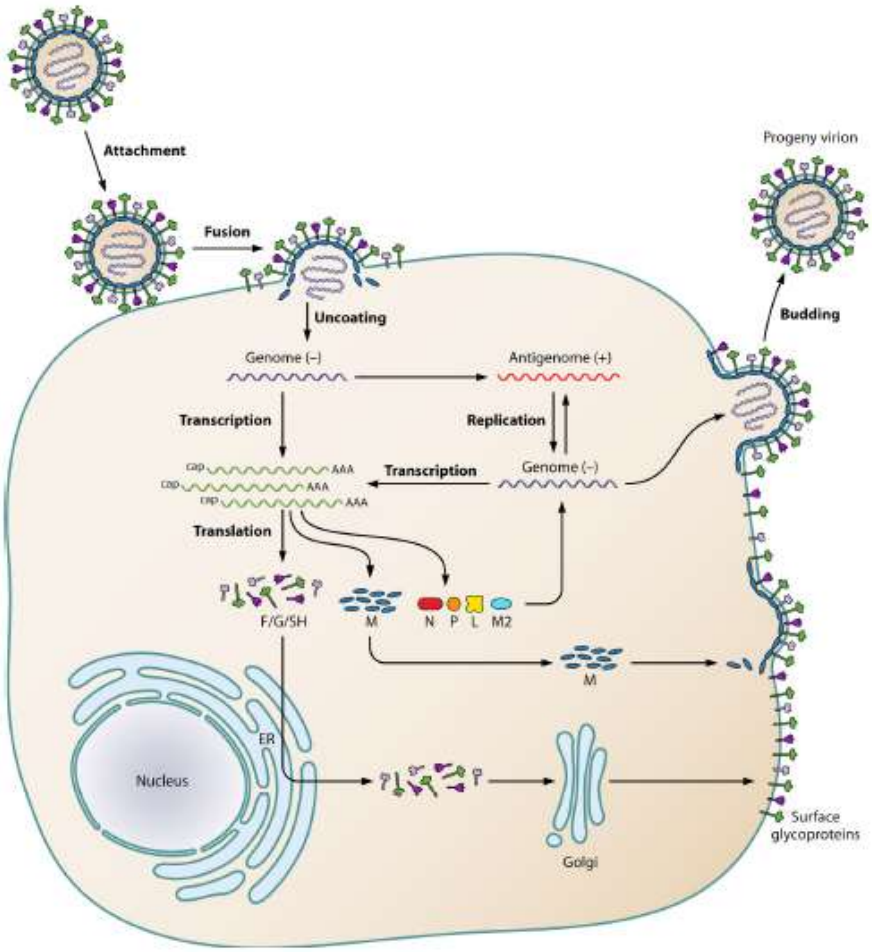


Figura 8. Ciclo de replicación del MPVH. El ciclo inicia con la unión de la proteína G a la célula hospedera, seguida por la fusión por parte de la proteína F. Después de la unión del virión a la membrana plasmática, el ARN es liberado para dar inicio a los procesos de trascripción y replicación.. A partir del ARN genómico se sintetizan los ARN m necesarios para la síntesis de proteínas. La replicación se inicia con la síntesis de antigenomas, que serán templado para la síntesis de nuevos genomas completos. Después de la traducción, las proteínas M, N, P y L, son trasportadas intracelularmente a la membrana plasmática y las proteínas de superficie F, G y SH; son trasportadas del retículo endoplasmático al aparato de Golgi y de ahí a la membrana plasmática, como parte de su maduración. Finalmente, nuevos viriones son ensamblados y subsecuentemente liberados mediante el proceso de brote. (Imagen tomada de Schildgen et al., 2011).

6. Estacionalidad de VRSH y MPVH

Las epidemias de VRSH ocurren anualmente durante los meses de invierno en climas templados, mientras que los brotes en climas tropicales se asocian con la temporada lluviosa. El clima en Panamá es similar a los países tropicales de América Central. La

estación lluviosa es entre mediados de abril a diciembre con altos porcentajes de humedad, el resto del año se considera como estación seca y la humedad durante el día es menor. Estudios en Panamá sobre epidemiología de las infecciones respiratorias, mostró una patrón de estacionalidad para la infección con VRSH. El primer pico de la enfermedad se observa en mayo y aumenta con la intensificación de las lluvias con picos entre agosto y noviembre (Weber et al., 1998, Cane et al., 2001). La estacionalidad de MPVH ha sido reportada con picos principalmente durante los meses de invierno, similar o después de la temporada epidémica del VRSH (Boivin et al., 2002); también ha mostrado picos estacionales durante primavera (Mackay et al., 2004). En Centroamérica la estacionalidad no está claramente definida. Aunque varios estudios han reportado patrones de estacionalidad principalmente durante la estación lluviosa (Ramírez et al., 2014), también se ha visto la ocurrencia de picos menores en diferentes meses durante el año (García et al., 2012; McCracken et al., 2014) .

7. Epidemiología Molecular

7.1. VRSH

Basado en la variabilidad antigénica y genética de la proteína G, el VRSH se ha dividido en dos grupos: VRSH-A y VRSH-B (Anderson et al., 1985; Mufson et al., 1985). Para el genotipado de VRSH, la segunda región hipervariable del gen G se ha utilizado comúnmente debido a su mayor grado de divergencia, además, el análisis de esta región es capaz de reflejar la variabilidad genética global (Gail et al., 1998). Hasta la fecha, los estudios epidemiológicos moleculares sobre esta región han reportado varios genotipos para cada grupo antigénico. Mediante análisis filogenéticos Peret et al., 1998; observó diferentes

clados , a los que denominó por primera vez como genotipos GA o GB dependiendo del grupo antigénico al que correspondían, reportando posteriormente, la circulación de 5-7 genotipos de VRSH (Peret et al., 2000), (Fig.9).

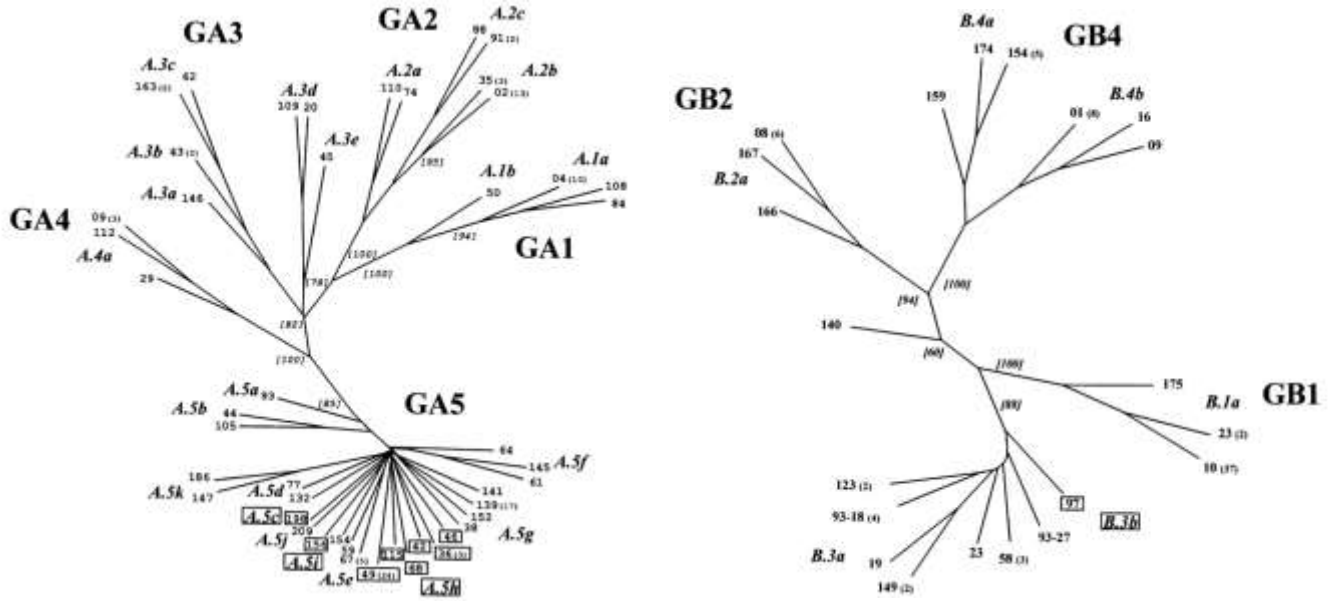


Figura 9. Árboles del VRSH, enraizados mediante el método probabilístico de máxima verosimilitud. Muestran las relaciones filogenéticas para el grupo VRSH- A (a) y B (b). Los números en cursiva entre corchetes en el interior principal de las ramas muestran el soporte del análisis (utilizando 100 réplicas). Los números entre paréntesis en los nodos terminales corresponden a número de secuencias idénticas. Los números en negrita en cursiva (a, 1a-5k; b, 1a-4b) indicar subtipos. Las cajas cuadradas se utilizan para asociar secuencias de virus con el correspondiente subtipo cuando sea necesario. Se indican genotipos con letras mayúsculas (GA1-GA5 y GB1-GB4). Las longitudes de rama no están dibujadas.(imagen tomada de Peret et al., 1998)

Hasta la fecha, para VRSH-A se han reportado 15 genotipos (GA1 a GA7, SAA1, SAA2, NA1-NA4, CB-A), entre ellos un nuevo genotipo ON1 que presenta una duplicación de 72 nucleótidos en el gen G (Peret et al., 1998, Peret et al., 2000, Venter et al., 2001, Shobugawa et al., 2009, Baek et al., 2012, Eshaghi et al., 2012, Ivancic). Para el VRSH-B, se han reportado 26 genotipos (GB1 a GB5, SAB1 a SAB4, URU1, URU2, CB- B, CB-1 y BA1 a BA13) (Peret et al., 1998;Venter et al., 2001; Baek et al., 2012; Trento et al., 2006; Blanc et al., 2005; Dapat et al., 2010; Arnott et al., 2011; Cui et al., 2013; Trento et al., 2010; Gimferrer et al., 2015; Khor et al., 2013). Generalmente, las cepas A son

predominantes sobre las cepas del grupo B en los años epidémicos, sin embargo, ambas pueden co-circular dentro de la misma estación y la misma comunidad, con uno o dos genotipos dominantes reemplazados en años sucesivos (Hendry et al., 1986; Peret et al., 1998; Trento et al., 2010; Hall et al., 1990). La presencia de duplicaciones en el gen G como mecanismo de variabilidad genética se observó por primera vez en VRSH en cepas aisladas en Buenos Aires, Argentina. En estos aislados de VRSH-B se observó una duplicación de 60 nucleótidos en la porción C-terminal del gen G. Este nuevo linaje viral dio lugar a un nuevo genotipo, denominado BA, el cual circuló a partir de 1999 y compartió un antepasado común datado en 1998 (Trento et al., 2006; Trento et al., 2010). De acuerdo con estudios epidemiológicos globales, el genotipo BA se ha propagado rápidamente a diferentes países del mundo, resultando el genotipo predominante de HRSV-B desde su emergencia en 1999 (Trento et al., 2010; van Niekerk y Venter, 2011; Pretorius et al., 2013; Khor et al., 2013).

Estudios previos han demostrado la importancia de VRSH como causante de las infecciones respiratorias agudas (IRA) en Centroamérica, siendo este virus responsable del 6,9-33% de la IRA en niños y del 5 al 6% en adultos (Mccracken et al., 2010; Schlaudecker et al., 2013). A pesar de esto, es escaso el conocimiento sobre la epidemiología molecular de este virus en la región centroamericana. El único reporte de patrones genéticos del VRSH durante 1995-2000 en la región caribeña es de Cuba (Valdés et al., 2004).

Los informes de 2011 a 2014 del Programa de Vigilancia de Influenza Panameña y otros Virus Respiratorios mostraron que la frecuencia de VRSH en muestras nasofaríngeas de niños menores de 5 años con IRA osciló entre 20-40%.

7.2. MPVH

Se han descrito dos linajes principales para MPVH, A y B (Fig.10); basados en diferencias en los títulos de neutralización de virus y en los análisis filogenéticos de genes N, P, M, F y G (Van den Hoogen et al., 2001, Peret et al., 2002).

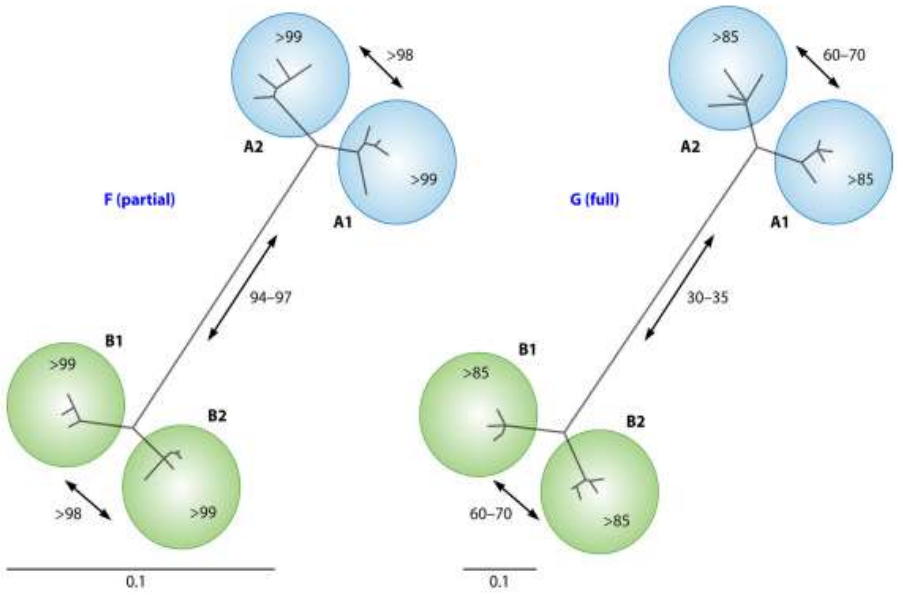


Figura 10. Árboles filogenéticos para genes de fusión (F) y de unión (G) de aislamientos seleccionados de MPVH. Para cada uno de los cuatro linajes genéticos , cuatro aislados representativo fueron seleccionadoss y se generaron árboles de máxima probabilidad para el gen G (derecha) y para 451 nucleótidos del gen F (izquierda). Los números en los árboles representan porcentajes de identidad de aminoácidos entre los aislados de virus (imagen tomade de Schildgen et al., 2011).

Además, cada linaje se divide en dos subgrupos genéticos (A1 y A2 en el linaje A, y B1 y B2 en el linaje B) (van den Hoogen et al., 2004). Posteriormente, el subgrupo A2 se ha dividido en dos clados (A2a y A2b) (Huck et al., 2006). Después de su descubrimiento en el 2001 en Holanda, el MPVH ha sido reportado en otros países europeos como Inglaterra (Stockton et al., 2002), Alemania (Huck et al., 2006), Francia (Freymouth et al., 2003), Finlandia (Jartti et al., 2002), España (Vicente et al., 2003), Italia (Lo Pesti et al., 2011); en Australia (Mackay et al, 2004), Asia (Peiris et al., 2003; Choudhary et al., 2013; Chung et

al., 2006; Zhang et al., 2012), Norteamérica (Peret et al. 2003). En Sudamérica se han realizado estudios en Brasil (Cuevas et al., 2003), Argentina (Galiano et al., 2004), Uruguay (Mirazo et al., 2005; Pizzorno et al., 2010) y Chile (Luchsinger et al., 2005). Además, el MPVH se ha descrito en estudios realizados en Centroamérica (Garcia et al., 2012), y en países específicos, como Costa Rica (Ulloa-Gutiérrez et al., 2009), Honduras (Schlaudeker et al., 2012) y Guatemala (McCracken et al., 2014; Verani et al., 2013). En Panamá, a finales del 2010, el programa de vigilancia para Influenza y otros virus respiratorios, incluyó dentro de su panel de detección, el diagnóstico para MPVH. De acuerdo a los datos recolectados para este virus, MPVH ha mostrado una frecuencia de 1.3-13.9% en niños $\leq$ 5 años de edad entre el 2011 al 2012.

8. Vacunas

8.1. VRSH

El desarrollo de vacunas contra el VRSH ha progresado durante medio siglo, dando un extraordinario número de candidatos vacunales. Las proteínas que han sido estudiadas con más frecuencia son F y G, ya que cada una de ellas aparece en la partícula nativa de VRSH y son blancos importantes de anticuerpos neutralizantes y efectoras de las células T. De estas dos proteínas, la F se conserva mejor y es quizás la más adecuada para inducir linfocitos que reaccionan de forma cruzada con antígenos de VRSH A y B (Hurwitz, 2011). Los productos vacunales estudiados hasta el momento incluyen: virus muertos, proteínas del virus purificada (Murphy et al ., 1990), virus atenuado (Karron et al., 2005), nanopartículas, partículas similares a virus (VLP: partículas que imitan la conformación del virus nativo, pero carecen de material genético), virosomas basados en envolturas virales de

VRSH en membranas sin nucleocápside o ácidos nucleicos (Stegmann et al., 2010), subunidades recombinantes (Power et al., 2001), vectores competentes portadores de genes de HRSV, vectores deficientes en replicación portadores de genes de HRSV y plásmidos de ADN que expresan las proteínas F o G (Li et al., 1998; Bembridge et al., 2000; Kohlmann et al., 2009; Hurwitz, 2011; Kim y Chang, 2012).

8.2. MPVH

Desde el descubrimiento de los metapneumovirus, varios equipos se han dedicado al desarrollo de una vacuna específica para MPVH. Varias estrategias han sido investigadas, en base a virus inactivados (de Swart et al., 2007), virus vivos atenuados (Buchholz et al., 2006), proteínas recombinantes (Biacchesi et al., 2004.); y vacunas ADN, que consiste en inyectar plásmido que codifica una proteína viral, no permitiendo replicación viral y por lo tanto ningún riesgo de enfermedad infecciosa (Vuola et al., 2005). El principal desafío que enfrenta el desarrollo de una vacuna contra el MPVH es la dificultad de inducir una fuerte respuesta inmune que sea además de larga duración. Aunque ninguno de los enfoques anteriores ha sido probado en ensayos clínicos, muchos de ellos son potencialmente interesantes y requieren un mayor desarrollo (Feuillet et al., 2012).

JUSTIFICACIÓN

La vigilancia mundial en curso y la caracterización de las cepas circulantes de VRSH y MPVH son necesarias principalmente para contribuir al desarrollo de vacunas. Los estudios que muestran las cepas circulantes y la variabilidad genética de estos virus son escasos en la región centroamericana. A través del Programa de Vigilancia Epidemiológica para Influenza y otros Virus Respiratorios en Panamá, se ha logrado conocer la frecuencia de ambos virus en el país. Hasta la fecha, y en concordancia con lo que ocurre a nivel mundial la mayor frecuencia de casos corresponde a VRSH, sin embargo se carece de información sobre la variabilidad antigénica y genética de este virus en Panamá. El MPVH, al ser un virus descrito hace relativamente poco tiempo en comparación con el VRSH no estaba incluido dentro del diagnóstico de rutina y se conoce menos aún sobre este virus. A finales de 2010 se incluyó este agente infeccioso en el Programa de Vigilancia Epidemiológica para Influenza y otros Virus Respiratorios y a partir de entonces, el número de muestras positivas para este virus ha ido en aumento En base a estas consideraciones, nos planteamos estudiar la variabilidad genética y antigénica de HRVS y HMPV en Panamá en el período 2008 -2012, a partir de muestras de niños $\leq$ 5 años.

OBJETIVOS

General:

Caracterizar y analizar la diversidad de cepas de VRSH y MPVH aisladas en Panamá a partir de hisopados nasofaríngeos provenientes de niños ≤ 5 años con IRAs.

Específicos:

- Aislar cepas de VRSH a partir de hisopados nasofaríngeos provenientes de niños menores de 5 años con IRAs.
- Amplificar y secuenciar el gen G de las cepas de VRSH circulantes durante los años 2008 al 2012, en Panamá.
- Realizar análisis de variabilidad genética de VRSH, mediante la construcción de árboles filogenéticos de las secuencias del gen G.
- Analizar la variabilidad antigénica de cepas de VRSH-A, mediante un panel de anticuerpos monoclonales contra la glicoproteína G.
- Amplificar y secuenciar el gen N de cepas de MPVH a partir de hisopados nasofaríngeos provenientes de niños menores de 5 años con IRAs.
- Realizar análisis de variabilidad genética de MPVH, mediante la construcción de árboles filogenéticos de las secuencias del gen N.

HIPÓTESIS:

Las cepas de VRSH y MPVH que circulan en Panamá se caracterizan por una marcada diversidad genética y antigénica, al igual que lo reportados para el resto del mundo

CAPÍTULO I

CAPÍTULO I

CARACTERIZACIÓN GENÉTICA Y ANTIGÉNICA DE CEPAS DEL VRSH GRUPO A, AISLADAS EN PANAMÁ ENTRE 2008-2012.

CARACTERIZACION GENÉTICA:

Las cepas del grupo A de VRSH son en general predominantes sobre las cepas del grupo B en sucesivas epidemias. Sin embargo, ambos pueden co-circular en la misma estación y comunidad con uno o dos genotipos dominantes (Peret et al., 2000; Trento et al., 2010). Hasta la fecha, para VRSH-A se han reportado 15 genotipos (GA1 a GA7, SAA1, SAA2, NA1-NA4, CB-A), entre ellos un nuevo genotipo ON1 fue reportado en el 2012 (Peret et al., 1998, Peret et al., 2000, Venteret al., 2001, Shobugawa et al., 2009, Baek et al., 2012, Eshaghi et al., 2012). El nuevo genotipo ON1, se observó por primera vez en cepas de Canadá del 2010 y se caracteriza por la inserción de 72 nucleótido en la región C-terminal de la glicoproteína de unión (G). Esta inserción se deduce en una duplicación de 23 aminoácidos, con 7 sitios de O-glicosilación potenciales que incluyen tres sitios de O glicosilación en los residuos 270, 275 y 283 (Eshagji et al., 2012). Diferentes trabajos realizados en varios países han reportado el rápido reemplazo y predominio del genotipo ON1 a nivel mundial (Agoti et al., 2014; Tabatabai et al., 2014; Pierangeli et al., 2014; Duvvuri et al., 2015; Ivancic-Jelecki et al., 2015). En Panamá, sólo se conocia la frecuencia del VRSH, sin embargo, se desconocen los genotipos circulantes. Por lo que nos propusimos en esta tesis la caracterización molecular de cepas del VRSH de Panamá provenientes de niños menores de 5 años durante el período 2008-2012.

Materiales y Métodos:

Muestras Clínicas

El Instituto conmemorativo Gorgas de Estudios de la Salud (ICGES), de Panamá, cuenta con un programa de vigilancia epidemiológico para influenza y otros virus respiratorios y recibe muestras respiratorias de 18 instalaciones de salud distribuidos en el país. Para este estudio fueron utilizadas muestras con resultados positivos para el VRSH de niños menores de 5 años con IRAB colectadas durante julio 2008 a noviembre del 2012 fueron utilizadas

para este estudio. El protocolo para esta investigación fue sometido y aprobado por el Comité de Bioética del ICGES.

Extracción de ARN y RT-PCR

El ARN fue extraído de 140 µl de hisopados nasofaríngeos utilizando el kit de extracción QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) siguiendo las instrucciones del fabricante. Una RT-PCR fue realizada seguida por una PCR anidada para amplificar 1,115 pares de bases (pb) del gen de la proteína G y una pequeña porción del gen de la proteína F del VRSH, mediante el uso de un kit comercial de amplificación de QIAGEN One-Step RT-PCR (QIAGEN, Hilden, Germany) agregando 5 µl de ARN y usando los cebadoresLG5+(5´-GGATCCCGGGGCAAATGCAAACATGTCC-3´) y F164-(5´GTTATGACACTGGTATACCAACC-3) (Parveen et al., 2006). Las condiciones de temperatura para la RT-PCR fueron de: 45 mini a 48°C, 15 min. a 95°C, seguido de 45 ciclos a 95°C por un1 min., 54°C por 1 min., 68°C por 2 min., y una extensión final de 68°C por 5 min.. Para la PCR anidada, se utilizó el kit Taq-PCR Master Mix (QIAGEN, Germany) utilizando los cebadores: LG1+ (5´-GGGGCAAATGCAAA-3´) y LGF- (5´-TTTGCTAACTGCACTGCATGT-3´) agregando 2 µl del producto de la RT-PCR. Las condiciones de temperaturas fueron de 5 min a 95°C, seguido de 35 ciclos a 94°C por 1 min, 54°C por 1 min, 72°C por 1 min, con una extensión final de 72°C por 5 min. El producto amplificado fue sometido a electroforesis en gel de agarosa al 1.5 %, teñido con bromuro de etidio y visualizado bajo luz ultravioleta.

Secuenciación de ADN

Los productos de la PCR anidada fueron purificados usando el kit QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Ambas hebras fueron secuenciadas usando el kit BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) utilizando los mismos cebadores de la PCR anidada. La secuenciación fue realizada en el equipo 3130xl genetic analyzer (Applied Biosystems). Las secuencias obtenidas fueron editadas utilizando el software Sequencher 5.4.5. y depositadas en el GenBank con números de acceso: KF300951 to KF301020.

Análisis Filogenético

Las secuencias parciales del gen G fueron alineadas con secuencias representantes de cada genotipo reportado utilizando el software BioEdit v7.0.9.0. El análisis filogenético incluyó 96 secuencias, de las cuales 49 correspondieron a cepas panameñas y 47 a secuencias de referencias representativas de los genotipos. La cepa prototipo A2 (JX198138) de 1981, fue utilizada como grupo externo. La relación filogenética se reconstruyó bajo el criterio de máxima verosimilitud. El modelo de sustitución nucleotídica fue GTR+G (gamma shape=0,60). El soporte estadístico de los nodos se estimó mediante aLRT (approximate likelihood ratio test). Se muestran únicamente los soportes significativos para los nodos más relevantes.

Deducción de Amino ácidos

La secuencia deducida de aminoácidos (parcial) derivó de las secuencias de nucleótidos obtenidas del gen de la glicoproteína G y se dedujo utilizando el software BioEdit v7.0.9.0. Se analizaron las secuencias deducidas de aminoácidos desde la posición 210 hasta finalizar la región C – terminal del ectodominio de la proteína G, incluyendo el codón de terminación.

Las cepas panameñas del VRSH-A sin inserción de 72 nucleótidos fueron comparadas con la cepa prototipo A2 (JX198138) del VRSH-A aislada en 1961 y las cepas panameñas que contenían la inserción de 72 nucleótidos se analizaron en relación a la cepa ON67-1210A de Canadá aislada en 2010 (JN257693)

Resultados

Fueron seleccionados un total de 122 hisopados nasofaríngeos positivos para VRSH. 103 fueron amplificados para el gen G y de estos, 75 fueron secuenciadas. 60 secuencias provinieron de niños hospitalizados (80%, 60/75) y 15 de pacientes ambulatorios. 51 secuencias (68%) correspondieron al VRSH-A y 24 al VRSH- B. Ambos, VRSH A y VRSH-B co-circularon cada año, con predominancia del VRSH-A, a excepción del año 2008 en que el VRSH-B fue más predominante. De las secuencias del VRSH-A analizadas,

una correspondió a la epidemia 2008, ocho al 2010, veintiséis al 2011 y catorce al 2012. De las secuencias de VRSH-B analizadas, 13 correspondieron a la epidemia 2008, una a 2010, 9 a 2011 y una al año 2012.

Análisis genético y filogenético del VRSH-A

El análisis genético de las cepas panameñas del VRSH-A mostró 42 secuencias que incluyeron la inserción de 72 nucleótidos reportada en cepas de Canadá de 2010 pertenecientes al nuevo genotipo ON1. Sólo Siete secuencias no mostraron esta inserción y correspondieron a las epidemias 2008 (N=1, PAN1A/08), 2010 (N=4, PAN2A/10, PAN7A/10, PAN8A/10 y PAN9A/10), 2011 (N=1, PAN33A/11) y 2012 (N=1, PAN42A/12). Las cepas panameñas que mostraron la inserción de 72 nucleótidos, fueron mayoritarias a partir de la epidemia 2011. Cuatro cepas correspondieron a la epidemia 2010 (PAN3A/10, PAN4A/10, PAN5A/10 y PAN6A/10), veinticinco a 2011 (PAN10A-a PAN36A/10) y trece a 2012 (PAN37-42/12 y PAN43A a 46A, 47A a 51A/12). El análisis filogenético mostró que las cepas pertenecen mayoritariamente al genotipo ON1, mientras que un grupo minoritario está emparentado con cepas previamente reportadas como NA1, que carecen de la inserción de 72 nucleótidos. El genotipo ON1 es un grupo homogéneo, presentando una homología intra grupo del 99% y forman un grupo monofilético, con un soporte estadístico de 0,92. (Fig. 1).

El genotipo NA1 no aparece como grupo monofilético en nuestro análisis, sin embargo, las cepas panameñas asignadas a este genotipo poseen una homología nucleotídica del 97% en la región analizada (p-dist=0.03).

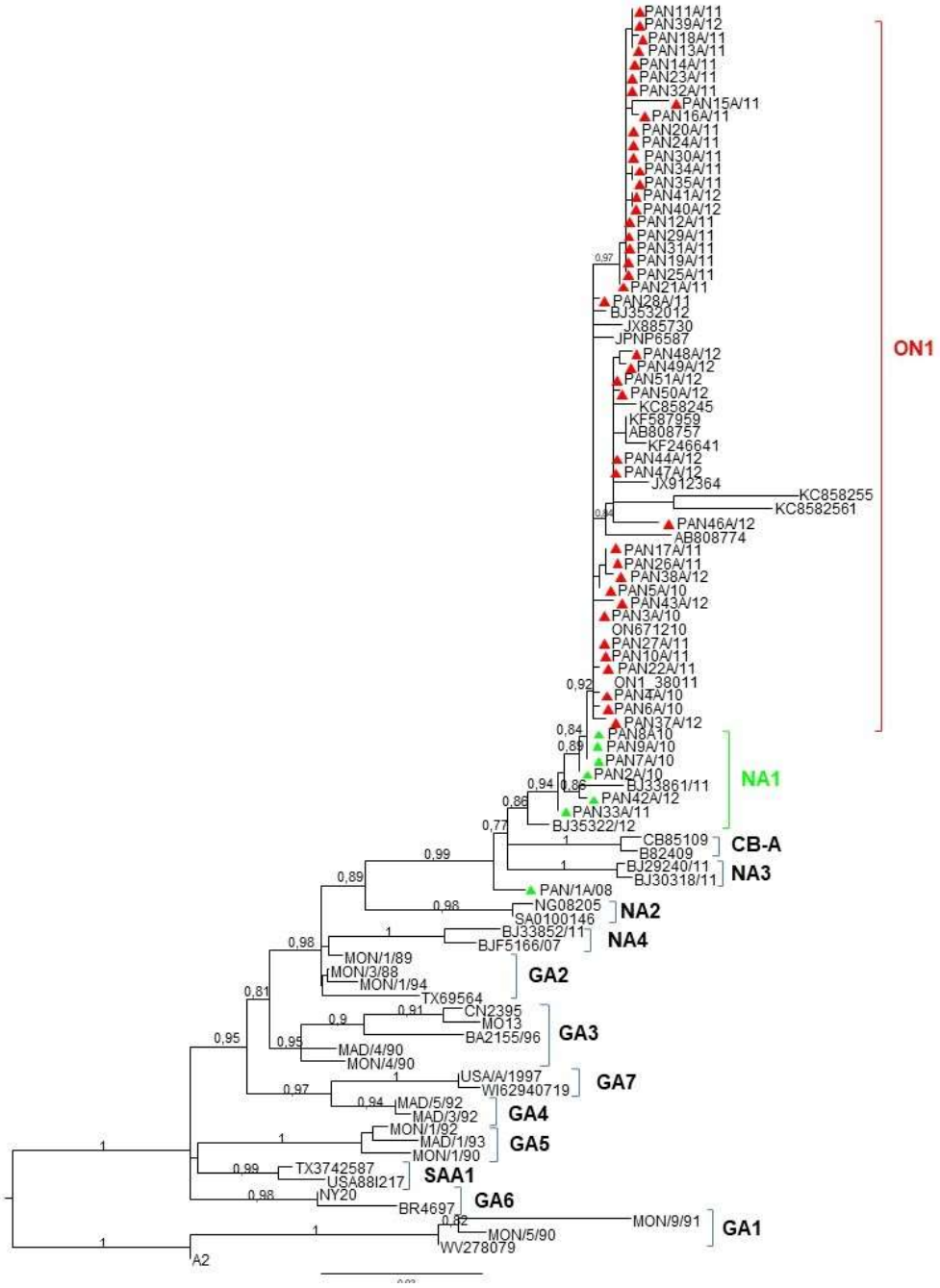


Figura 1. Árbol filogenético del VRSH-A utilizando el gen G de cepas Panameñas de 2008-2012. El análisis incluyó 49 secuencias Panameñas y 47 secuencias de referencia representativas de los genotipos reportados. La filogenia, el modelo de sustitución nucleotídica, así como los soportes estadísticos fueron estimados utilizando el software PhyML v 3.0. La filogenia se reconstruyó bajo el criterio de máxima verosimilitud. El modelo de sustitución nucleotídica fue GTR+G (gamma shape=0,60). El soporte estadístico de los nodos se estimó mediante aLRT (approximate likelihood ratio test). Se muestran únicamente los soportes significativos para los nodos más relevantes. Los triángulos verdes indican las cepas sin inserción de 72 nucleótidos y los rojos marcan las cepas que incluyen la inserción de 72 nucleótidos.

Secuencia de amino ácidos de la proteína G de las cepas del VRSH-A

La longitud predicha de la proteína G para todas las cepas panameñas de VRSH-A fue de 297 amino ácidos para las cepas sin inserción de 72 nucleótidos y presentaron sitios potenciales de N glicosilación conservados en las posiciones 237, 251 y 294. Las cepas con inserción de 72 nucleótidos tuvieron una longitud de 321 amino ácidos. También presentaron sitios probables de O-glicosilación conservados en las posiciones 267, 270, 275 y 283 con serina y treonina en las posiciones 227, 231, 235 y 282; previamente reportados (Collins et al., 2001). Se observaron dos sitios potenciales de N- glicosilación en las posiciones 237 y 318 de 46 de las 49 cepas panameñas. La cepa PAN1A/08 mostró una mutación en la posición 237 y las cepas PAN28A/11, PAN34A/11 y PAN35A/11 en la posición 318 (Tabla 1, a y b).

Se observaron 22 sustituciones de amino ácidos en las cepas sin inserción comparadas con la cepa prototipo A2 (Tabla 1, A). La mayoría de estas sustituciones (S222P, P226L, E233K, I244R, L258H, M262E, F265L, S269T, P274L, S280Y, P286L, P289S, P292S, P293S, P296T y R297K) se encontraron en todas las cepas panameñas sin inserción (PAN1A/08, PAN2A/10, PAN7A/10, PAN8A/10, PAN9A/10, PAN33A/11 y PAN42A/12); a excepción de las 4 últimas posiciones (292, 293, 296 y 297) para la cepa PAN9A/10, ya que no se logró obtener la secuencia final que incluyera el codón de terminación de la proteína. Los cambios K210I, K221R, N237D y E271K, sólo se observaron en la cepa PAN1A/08 y el cambio E271K se observó en todas las cepas panameñas sin inserción a excepción de la cepa PAN1A/08. Por último, el cambio S290L, sólo se observó en las cepas del 2010 (PAN2A, PAN7A, PAN8 y PAN9). Prácticamente todas las sustituciones de amino ácidos se encontraron en los genotipos previamente reportados (SAA1, GA1-GA7, NA1-NA4 y CB-A), con la excepción de los cambios K221R y E271K, que sólo se observaron en la cepa panameña, PAN1A/08 (Tabla 2).

Al comparar las cepas panameñas con la cepa ON67-1210A, (genotipo ON1, el cual posee la inserción de 72 nucleótidos), encontramos 14 cambios de amino ácidos (P217L, G232R, L247P, K253N, Y273H, L274P, Y280H, H290L, T292I, L298P, P300S, Y304H, N318D Y N318Y). (Tabla1B). El cambio P217L se observó sólo en la cepa PAN46A/12. El cambio G232R, se observó en las cepas PAN11A-PAN16A/11, PAN18A/11, PAN19A/11, PAN20A, PAN23A/11, PAN24A, PAN25A/11, PAN29A/11, PAN30A/11, PAN31A/11,

PAN32A/11, PAN34A/11, PAN35A/11, PAN39A/12, PAN40A/12 y PAN41A/12. El cambio L247P, se observó en las cepas PAN15A/11, PAN17A/11, PAN26A/11 Y PAN38A/12. El cambio K253N, se observó sólo en la cepa PAN50A/12. El cambio Y273H, se observó sólo en la cepa PAN15A/11. El cambio L274P, estuvo presente en las cepas PAN18A/11, PAN44A/12, PAN46A-PAN51A/12. Y280H, sólo se observó en la cepa PAN46A/12. H290L sólo se observó en la cepa PAN48A/12. T292I en la cepa PAN43A/12. L298P en las cepas PAN11A711, PAN13A/11, PAN18A/11, PAN39A/12, PAN44A/12, PAN46A/12, PAN47A/12, PAN48A/12, PAN49A/12, PAN50A/12 Y PAN51A/12. P300S sólo en la cepa PAN48A/12. Y304H se observó en las cepas PANA44A/12 y PAN46A/12-PAN51A/12. N318D sólo en PAN28A/11. N318D sólo en PAN28A/11 y el cambio N318Y en la cepa PAN34 y PAN35A/12. La mayoría de los cambios de amino ácidos descritos, han sido previamente reportados en cepas del 2006-2014, principalmente de USA, Alemania, Italia, Korea, Japón, China, Filipinas, Vietnam, Pakistán y Kenia. A excepción de los cambios G232R, K253N, N318Y y N318D, que sólo fueron encontrados en las secuencias de las cepas de Panamá. (Tablas 1 y 2).

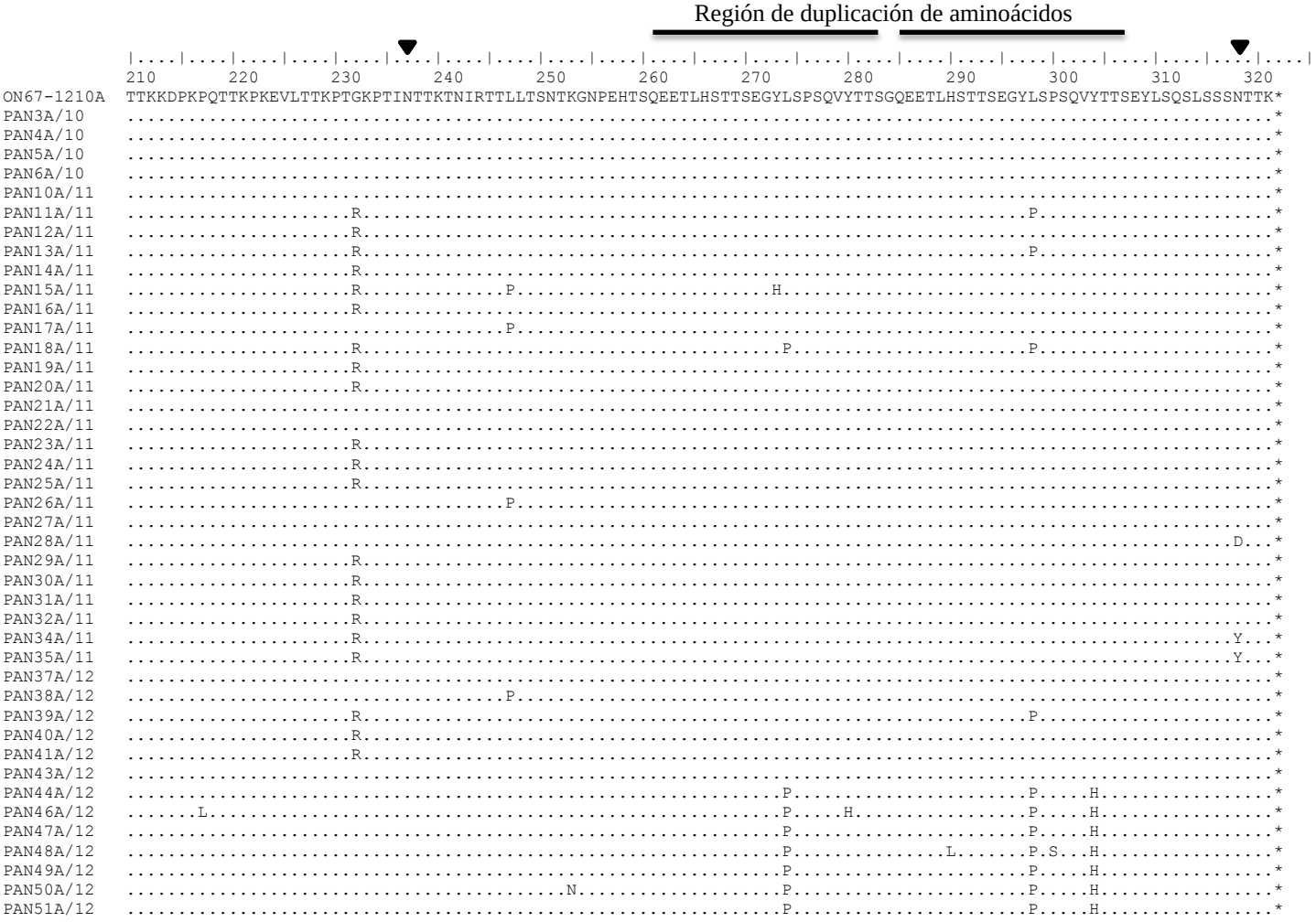
Tabla 1. Deducción de amino ácidos alineadas con secuencias parciales del gen G de VRSH-A

(A)

```
                          |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                          210       220       230       240       250       260       270       280       290       300

A2                        TTKKDPKPQTTKSKEVPTTKPTEEPTINTTKTNIITTLLTSNTTGNPELTSQMETFHSTSSEGNPSPSQVSTTSEYPSQPSSPPNTPRQ
MON-5-90/GA1              ..............................................R.........................I.............S.*
MON-3-88/GA2              .....L......P...L......K..........R.............H...K..L...T..........Y.....L..SL..S..TN*
CN2395/GA3                --...I......Q...L...L..K........K.R.....T.......H...K..L.........L.....YP....L..SP..S..TNL
MAD-5-92/GA4              .....F......P...L......K..........K.....T.......H...K..L....P.........Y ........P..S..TD*
MON-1-90/GA5              .....L......P..AL......K....I..P..R.....NS......H...E..L........I.....Y.....L...P..S.ITN.
MON-4-90/GA6              .....L......Q..........K..........S.....T.......H...K..L.....D........Y ....L..SP..S..TNL
USA/A/1997/GA7            .....L......P...L......K.....R....G.....T.......Y...K..L....P.........Y.........P..SY.TNE
USA/88I-217A/SAA1         I....I......P..........KS.........R.....N.......H...K..L..............Y.....L...P..S..TNW
CB851/09/CB-A             .....S......P...L......KQ..D......R.............H...GGALR..T....LG....Y.....L..SP..SS.THR
BJ/33861/11/NA1           ................L......K..........R.....F.......H...E..L...T...YL.....Y.....L..SP..S..TK*
SA01-00146/NA2            .....L......P...L......K..........R.......I.E.Q.H...K..L...T..........Y.....L..SL..S..T.W
BJ/29240/11/NA3           ............P...L......Q.........VR..........D..H...E..L...TF.........Y.....L..SP..S..TK*
BJ/33852/11/NA4           .....L......P..........Q...H.S..I.G.....F.......H...K..L...T....Q.....Y.....L..SL..S..TYY
PAN1A/08                  I..........RP...L......K...D......R.............H...E..L...T....L.....Y.....L..SP..S..TK*
PAN2A/10                  ............P...L......K..........R.............H...E..L...T.K.YL.....Y.....L..SL..S..TK*
PAN7A/10                  ............P...L.....GK..........R.............H...E..L...T...YL.....Y.....L..SL..S..TK*
PAN8A/10                  ............P...L.....GK..........R.............H...E..L...T...YL.....Y.....L..SL..SS.TK-
PAN9A/ 10                 ............P...L.....GK..........R.............H...E..L...T...YL.....Y.....L..SL--------
PAN33A/11                 ............P...L......K..........R.............H...K..L...T...YL.....Y.....L..SP..SS.TK*
PAN42A/12                 ............P...L......K..........R.............H...K..L...T...YL.....Y.....L..SP..SS.TK*
```

Los asteriscos (*) indican el codón de terminación los triángulos invertidos (▼) muestran sitios potenciales de N glicosilación



(A) Cepas de referencia sin la duplicación de 23 aminoácidos (Largo de la proteína, 297 aminoácidos) y cepas de Panamá sin inserción, comparadas con la cepa prototipo A2. (B) Cepa prototipo de referencia ON67-1210 y secuencias de Panamá con de la duplicación de 23 aminoácidos (Largo de la proteína 321 aminoácidos). La deducción del largo de proteína G se muestra a la derecha. En asterisco (*) se localiza el codón de terminación. Los asteriscos (*) indican el codón de terminación, los triángulos invertidos (▼) muestran sitios potenciales de N glicosilación, residuos idénticos están indicados por puntos.

Tabla 2. Sustituciones de aminoácidos en la glicoproteína G de las cepas panameñas VRSH-A sin inserción y con inserción de 72 nucleótidos.

Sustitución de amino ácidos	Cepas Panameñas	Genotipos de las cepas panameñas	País y año de cepa, previamente publicado	Genotipo previamente reportado	Referencia
T210I	PAN1A/08	NA1	USA (KJ723468)	SAA1/1988	Sometida directamente al GenBank Das et al., 2014, genoma completo.
P217L	PAN46A/12	ON1	China (BJ/F6116)	NA1	Cui et al., 2013
K221R	PAN1A/08	NA1	China (BJ/29236, BJ/27760)	NA1	Cui et al., 2013
S222P	Todas las cepas Panameñas sin inserción	NA1	USA 1988, 1997 Korea 2009 China, 2004-2006, 2011 Uruguay, 1988, 1990 España, 1992	SAA1, CB-A GA2,GA4. GA5, GA7, NA1-NA4	Baek et al., 2012 García., 1994 Lorenzi et al., 2012 Cui et al., 2013 Shobugawa et al., 2009
P226L	Todas las cepas Panameñas sin inserción	NA1	USA 1988, 1997 Korea 2009 China, 2004-2006, 2011 Uruguay, 1988, 1990 España, 1992	SAA1, CB-A GA2,GA4. GA5, GA7, NA1-NA4	Das et al., 2014 Baek et al., 2012 García., 1994 Lorenzi et al., 2012 Shobugawa et al., 2009 Cui et al., 2013
E232G	PAN7A/10, PAN8A/10 PAN9A/10	NA1	Korea 2008-2010, China 2012-2013, Japón 2002-2006, Filipinas 2008-2012, Vietnam 2009-2010, Alemania 2012-2013	NA1	Ren et al.,2014 Baek et al., 2012 Shobugawa et al., 2009 Ohno et al., 2013 Ha do el al., 2015 Tabatabai et al.,2014

G232R	PAN11A/11, PAN12A/10, PAN13A/11, PAN14A/12 PAN15A/11 PAN16A/10, PAN18A/11, PAN19A/12 PAN20A/11 PAN23A/10, PAN24A/11, PAN25A/12 PAN29A/11 PAN30A/10, PAN31A/11, PAN32A/12 PAN34A/12 PAN35A/11 PAN39A/10, PAN40A/11, PAN41A/11,	ON1			**Solamente en cepas de Panamá**
E233K	Todas las cepas Panameñas sin inserción	NA1	USA 1988, 1997 Korea 2009 China, 2004-2006, 2011 Uruguay, 1988, 1990 España, 1992	GA2-GA7 SAA1, CB-A NA1, NA2	Das et al., 2014 Baek et al., 2012 García., 1994 Lorenzi et al., 2012 Cui et al., 2013 Shobugawa et al., 2009
N237D	PAN1A/08	NA1	Japón 2006 Korea 2008-2010 Filipinas 2008-2012 Vietnam 2009-2010 Pakistan 2012 Alemania 2012-2013 China 2007-2010	NA1 CB-A	Shobugawa et al., 2009 Baek et al., 2012 Ohno et al., 2013 Ha do el al., 2015 Aamir et al., 2013 Tabatabai et al.,2014 Cui et al., 2013
I244R	Todas las cepas Panameñas sin inserción	NA1	USA 1988, 1997 Korea 2009 China 2004-2006, 2011 Uruguay, 1988, 1990 España, 1992	SAA1,CB-A GA2-GA5 NA1-NA3	Das et al., 2014 Baek et al., 2012 García., 1994 Shobugawa et al., 2009 Cui et al., 2013
L247P	PAN15A/11 PAN17A/11 PAN26A/11 PAN38A/12	ON1	Filipinas 2011 Italia 2010	NA1	Ohno et al., 2013 Pierangeli et al.,2014
K253N	PAN50A/12	ON1			**Solamente en cepa de Panamá**

L258H	All Panamanian strains without insertion	NA1	USA 1988, 1997 Korea 2009 China 2011 Uruguay, 1988, 1990 España, 1992	GA2-GA7 SAA1,CB-A NA1-NA3	Das et al., 2014 Baek et al., 2012 García., 1994 Shobugawa et al., 2009 Cui et al., 2013
M262E	Todas las cepas Panameñas sin inserción	NA1	Uruguay 1990 China 2004-2006, 2011	GA5 NA1,NA3	García., 1994 Shobugawa et al., 2009 Cui et al., 2013
F265L	All Panamanian strains without insertion	NA1	Uruguay 1988 USA 1988 Korea 2009 China 2004-2006, 2011 Uruguay, 1988, 1990 España, 1992	GA2-GA7 SAA1,CBA NA1-NA4	García., 1994 Das et al., 2014 Baek et al., 2012 Shobugawa et al., 2009 Cui et al., 2013
S269T	Todas las cepas Panameñas sin inserción	NA1	España, 1992 Korea 2009 China 2004-2006, 2011	GA2,CB-A NA1-NA4	García., 1994 Baek et al., 2012 Shobugawa et al., 2009 Cui et al., 2013
E271K	PAN1A/08	NA1	Italia 2010 China (BJ/34884)	NA1	Pierangeli et al.,2014 Cui et al., 2013
Y273N	PAN1A/08	NA1	Japón 2001-2006 Korea 2008-2010 Filipinas 2008-2012 China 2007-2010 Vietnam 2009-2010 Pakistán 2012	GA5 NA1 NA2 NA3 NA4 CB-A	Shobugawa et al., 2009 Baek et al., 2012 Ohno et al., 2013 Cui et al., 2013 Ha do el al., 2015 Aamir et al., 2013
N273Y	Todas las cepas Panameñas sin inserción (excepto para PAN1A/08)	NA1	China 2011	NA1	Cui et al., 2013
Y273H	PAN15A/11	ON1	Italia 2010-2011 Japón 2012-2013	NA1 ON1	Pierangeli et al.,2014 Hirano et al., 2014
P274L	Todas las cepas Panameñas sin inserción	NA1	Canadá, 1994-1995 Korea 2009 China 2004-2006, 2011	GA3,CB-A, NA1	Peret et al., 2000 Baek et al., 2012 Shobugawa et al., 2009 Cui et al., 2013
L274P	PAN18A/11 PAN44A/12 PAN46A/12 PAN47A/12 PAN48A/12 PAN49A/12 PAN50A/12 PAN51A/12	ON1	Vietnam 2009-2010 Italia 2010-2011 Senegal 2013-2015 Alemania 2012 Pakistán 2012	NA1 ON1	Hado et al., 2015 Pierangeli et al., 2014 Fall et al.,2016 Prefiert et al., 2012 Aamir et al.,

S280Y	Todas las cepas Panameñas sin inserción	NA1	USA 1997 Canadá 1994-1995 Korea 2009 China 2011 Uruguay, 1988, 1990 España, 1992	GA2-GA7 CB-A NA1,NA2 y NA4	García., 1994 Peret et al., 2000 Baek et al., 2012 Lorenzi et al., 2012 Shobugawa et al. 2009 Cui et al., 2013
S280H	PAN46A/12	ON1	Japón 2001 Alemania 2011 Italia 2011 Senegal 2013-2014 China 2007-2010	GA5 NA1 NA3	Shobugawa et al., 2009 Tabatabai et al., 2014 Pierangeli et al., 2014 Fall et al., 2016 Cui et al., 2013
P286L	Todas las cepas Panameñas sin inserción	NA1	USA 1988, 1997 Canadá 1994-1995 Korea 2009 China 2004-2006, 2011 Uruguay, 1988, 1990 España, 1992	GA2,GA3 GA5,GA6 SAA1,CB-A NA1,NA2 y NA4	García., 1994 Peret et al., 2000 Das et al., 2014 Baek et al., 2012 Lorenzi et al., 2012 Shobugawa et al., 2009 Cui et al., 2013
P289S	Todas las cepas Panameñas sin inserción	NA1	USA 1988, 1997 España, 1992 Uruguay, 1988, 1990 Korea 2009 China 2004-2006, 2011	GA2,GA3 GA6,CB-A NA1-NA4	García., 1994 Peret et al., 2000 Baek et al., 2012 Lorenzi et al., 2012 Shobugawa et al., 2009 Cui et al., 2013
S290L	PAN2A/10, PAN7A/10, PAN8A/10 PAN9A/10	NA1	USA 1988, 1997 Korea 2009 China 2004-2006, 2011 Uruguay, 1988, 1990 España, 1992	GA2,NA2 NA4	García., 1994 Shobugawa et al., 2009 Cui et al., 2013
H290L	PAN48A/12	ON1	Japón 2005-2006 Pakistán 2012 Italia 2013	NA2 ON1	Shobugawua et al.,2009 Aamir et al.,2013 Pierangeli et al., 2014
T292I	PAN43A/12	ON1	China 2012-2013	NA1 ON1	Ren et al., 2014
P292S	Todas las cepas Panameñas sin inserción (excepto la PAN9A/10)	NA1	Korea 2009 China 2004-2006, 2011	CBA NA1, NA3	Baek et al., 2012 Shobugawa et al., 2009 Cui et al., 2013
P293S	Todas las cepas Panameñas sin inserción (excepto la PAN9A/10)	NA1	USA 1988, 1997 Korea 2009 China 2004-22006, 2011 Uruguay, 1988, 1990 España, 1992	GA2-GA7 SAA1,CB-A NA1-NA4	Das et al., 2014 Baek et al., 2012 García., 1994 Lorenzi et al., 2012 Shobugawa et al., 2009 Cui et al., 2013

P296T	Todas las cepas Panameñas sin inserción (excepto la PAN9A/10)	NA1	USA 1988, 1997 Korea 2009 China 2004-2006, 2011 Uruguay, 1988, 1990 España, 1992	GA2-GA7 SAA1,CB-A NA1-NA4	Das et al., 2014 Baek et al., 2012 García., 1994 Lorenzi et al., 2012 Shobugawa et al., 2009 Cui et al., 2013
R297K		NA1	China 2011	NA1, NA3	Shobugawa et al., 2009 Cui et al., 2013
L298P	PAN11A/11 PAN13A/11 PAN18A/11 PAN39A/12 PAN44A/12 PAN46A/12 PAN47A/12 PAN48A/12 PAN50A/12 PAN51A/12	ON1	Japón/ 2012 (AB698559) Kenia 2012-2013 Italia 2012-2013	ON1	Tanaka y Tanaka/no publicado Agoti et al., 2014 Bagnarelli/ no publicado
P300S	PAN48A/12	ON1	Korea 2009-2010 Filipinas 2008-2012	NA1 CB-A	Baek et al., 2012 Ohno et al., 2013
Y304H	PAN44A/12 PAN46A/12 PAN47A/12 PAN48A/12 PAN49A/12 PAN50A/12 PAN51A/12	ON1	Alemania 2012-2013 (WUE/16397/12) Kenia 2012-2013 Italia 2012-2013	ON1	Prefiert et al., 2013 Tabatabai et al., 2014 Agoti et al., 2014 Bagnarelli/ no publicado
N318Y	PAN34A/11 PAN35A/11	ON1			**Solamente en cepas de Panamá**
N318D	PAN28A/11	ON1			**Solamente en cepas de Panamá**

***Todas las cepas Panameñas sin inserción (PAN1A/08, PAN2A/10, PAN7A/10, PAN8A/10, PAN9A/10 PAN33A/11, PAN42A/12.**

Discusión

En este estudio se analizaron secuencias parciales del gen G de 49 cepas de VRSH del grupo A del 2008-2012, provenientes de hisopados nasofaríngeos de niños menores de 5 años en Panamá. Se observó la circulación de 2 genotipos, NA1 y ON1 previamente reportados (Shobugawa et al., 2009; Cui et al., 2013; Eshaghi et al., 2012). Siete cepas resultaron genéticamente similares a las cepas BJ33861/11 y BJ35322/12 del genotipo NA1. Estas cepas NA1 son basales con respecto al genotipo ON1 en la filogenia y carecen de la inserción de 72 nucleótidos en la proteína G. El resto de las cepas panameñas (42) se agruparon con el nuevo genotipo ON1, presentando la inserción de 72 nucleótidos que se

ubica en el tercio C-terminal de la proteína G del VRSH-A; la misma fue descrita por primera vez en cepas de Canadá del 2010. Coincidentemente, los primeros hallazgos de cepas con esta duplicación en Panamá se encontraron en cepas del 2010, también. Diferentes trabajos realizados en varios países han reportado el rápido reemplazo y predominio este genotipo ON1 en poco tiempo desde su aparición (Agoti et al., 2014; Tabatabai et al., 2014; Pierangeli et al., 2014; Duvvuri et al., 2015; Ivancic-Jelecki et al., 2015). Del total de cambios de amino ácidos observados, 11 han sido reportados como sitios bajo selección positiva: 226, 237, 244, 258, 262, 265, 273, 274, 286, 290 y 297 (Tan et al., 2012; Houspi et al., 2013; Pierangeli et al., 2014; Agoti et al., 2014; Kim et al., 2014). Diferentes autores hipotetizan que estos cambios de aminoácidos, podrían estar involucrados en la evasión de la respuesta inmune Botosso et al., 2009; Pretorius et al., 2013).

El análisis de la reactividad de cepas del VRSH-A, con un panel de anticuerpos monoclonales contra la proteína G obtenidos contra cepas 1957-1995 mostró que, a pesar de contar con una inserción de 23 aminoácidos, las cepas del genotipo ON1(20102012) mantienen la reactividad con dichos anticuerpos.

Esto indica que, a pesar de la variabilidad genética, las cepas actuales muestran un alto grado de conservación antigénica. Es necesario profundizar en estos estudios para conocer las implicancias en la evolución de esta proteína relevante para el proceso de infección en la célula hospedera (Trento et al., 2015).

CARACTERIZACIÓN ANTIGÉNICA:

MANUSCRITO: CONSERVATION OF G PROTEIN EPITOPES IN RESPIRATORY SYNCYTIAL VIRUS (GROUP A) DESPITE BROAD GENETIC DIVERSITY: IS ANTIBODY SELECTION INVOLVED IN VIRUS EVOLUTION?

Alfonsina Trento[1,2,‖], Leyda Ábrego[3,‖], Rosa Rodriguez-Fernandez[4], Maria Isabel González-Sánchez[4], Felipe González-Martínez[4], Adriana Delfraro[5], Juan M. Pascale[3], Juan Arbiza[5] and José A. Melero[1,2]

En este estudio se probaron anticuerpos monoclonales contra la proteína G de cepas pertenecientes a diferentes genotipos del virus respiratorio sincitial (VRSH), aislados en diferentes años epidémicos. Además se realizó un análisis evolutivo de diferentes genotipos del VRSH reportados a nivel mundial, hasta la actualidad. Los resultados obtenidos de ambos análisis genéticos y antigénicos, demostraron que a pesar del complejo patrón de diversificación del VRSH-A observado en los últimos 50 años y la dominancia temporal de genotipos a través del tiempo; esta variabilidad genética en el gen G podría no estar directamente relacionada con cambios antigénicos en dicha proteína. En este sentido, la deriva genética (al estilo de lo que se observa en virus como influenza A), no sería la principal fuerza que modula la extensa variabilidad genética observada en el gen G de VRSH.

---

[1]Unidad de Biología Viral, Centro Nacional de Microbiología and [2]CIBER de Enfermedades Respiratorias, Instituto de Salud Carlos III, Majadahonda, 28220 Madrid, Spain; [3]Departamento de Investigación en Virología, Instituto Conmemorativo Gorgas de Estudios de la Salud, Panamá; [4]Hospital General Universitario Gregorio Marañón, Madrid, Spain and [5] Sección Virología, Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo 11400, Uruguay. [‖]Igual Contribución.

*Corresponding Autor: José Antonio Melero. Unidad de Biología Viral, Centro Nacional de Microbiología. Instituto de Salud Carlos III, Majadahonda, 28220 Madrid, Spain. Tel: +34 918223908. Email: jmelero@isciii.es

JVI Journals.ASM.org

Conservation of G-Protein Epitopes in Respiratory Syncytial Virus (Group A) despite Broad Genetic Diversity: Is Antibody Selection Involved in Virus Evolution?

Alfonsina Trento,[a,b] Leyda Ábrego,[c] Rosa Rodriguez-Fernandez,[d] Maria Isabel González-Sánchez,[d] Felipe González-Martínez,[d] Adriana Delfraro,[e] Juan M. Pascale,[c] Juan Arbiza,[e] José A. Melero[a,b]

Unidad de Biología Viral, Centro Nacional de Microbiología,[a] and CIBER de Enfermedades Respiratorias, Instituto de Salud Carlos III,[b] Madrid, Spain; Departamento de Investigación en Virología, Instituto Conmemorativo Gorgas de Estudios de la Salud, Panamá, Panama[c]; Hospital General Universitario Gregorio Marañón, Madrid, Spain[d]; Sección Virología, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay[e]

ABSTRACT

Worldwide G-glycoprotein phylogeny of human respiratory syncytial virus (hRSV) group A sequences revealed diversification in major clades and genotypes over more than 50 years of recorded history. Multiple genotypes cocirculated during prolonged periods of time, but recent dominance of the GA2 genotype was noticed in several studies, and it is highlighted here with sequences from viruses circulating recently in Spain and Panama. Reactivity of group A viruses with monoclonal antibodies (MAbs) that recognize strain-variable epitopes of the G glycoprotein failed to correlate genotype diversification with antibody reactivity. Additionally, no clear correlation was found between changes in strain-variable epitopes and predicted sites of positive selection, despite both traits being associated with the C-terminal third of the G glycoprotein. Hence, our data do not lend support to the proposed antibody-driven selection of variants as a major determinant of hRSV evolution. Other alternative mechanisms are considered to account for the high degree of hRSV G-protein variability.

IMPORTANCE

An unusual characteristic of the G glycoprotein of human respiratory syncytial virus (hRSV) is the accumulation of nonsynonymous (N) changes at higher rates than synonymous (S) changes, reaching dN/dS values at certain sites predictive of positive selection. Since these sites cluster preferentially in the C-terminal third of the G protein, like certain epitopes recognized by murine antibodies, it was proposed that immune (antibody) selection might be driving the apparent positive selection, analogous to the antigenic drift observed in the influenza virus hemagglutinin (HA). However, careful antigenic and genetic comparison of the G glycoprotein does not provide evidence of antigenic drift in the G molecule, in agreement with recently published data which did not indicate antigenic drift in the G protein with human sera. Alternative explanations to the immune-driven selection hypothesis are offered to account for the high level of G-protein genetic diversity highlighted in this study.

Human respiratory syncytial virus (hRSV) is recognized as the major cause of severe acute lower respiratory tract infections (ALRI) in infants and young children worldwide (1). hRSV causes annual epidemics, and reinfections are common throughout life, although they are usually less severe than the primary infections. hRSV is also an important cause of morbidity and mortality in the elderly and in adults with cardiopulmonary disease or with an impaired immune system (2).

hRSV is an enveloped, nonsegmented, negative-sense RNA virus, classified in the genus *Pneumovirus* within the *Paramyxoviridae* family (for a recent review, see reference 3). The hRSV genome encodes 11 proteins, two of them being the major surface glycoproteins of the virus envelope. These are (i) the attachment (G) protein, which mediates binding of the virus to the cell surface (4), and (ii) the fusion (F) protein, which promotes fusion of the virus and cell membrane, allowing cell entry of the viral genome (5).

The G protein is a type II glycoprotein synthesized as a 32-kDa polypeptide precursor of 297 to 310 amino acids (aa), depending on the strain, and modified posttranslationally by the addition of several N-linked oligosaccharides and multiple O-linked sugar chains (6). The G-protein ectodomain (from residue 67 to the C terminus) has a central conserved region (aa 163 to 189) that includes four Cys residues (residues 173, 176, 182, and 186), and it is essentially devoid of potential glycosylation sites. This conserved region is flanked by two highly variable mucin-like segments, very rich in Ser and Thr, that are potential sites of O glycosylation. The extensive glycosylation of the G protein shapes its reactivity with both murine monoclonal antibodies (MAbs) (7) and human convalescent-phase sera (8).

hRSV isolates were originally classified into two antigenic groups (A and B) based on reactivity with hyperimmune serum

Received 20 February 2015 **Accepted** 11 May 2015

Accepted manuscript posted online 20 May 2015

Citation Trento A, Ábrego L, Rodriguez-Fernandez R, González-Sánchez MI, González-Martínez F, Delfraro A, Pascale JM, Arbiza J, Melero JA. 2015. Conservation of G-protein epitopes in respiratory syncytial virus (group A) despite broad genetic diversity: is antibody selection involved in virus evolution? J Virol 89:7776–7785. doi:10.1128/JVI.00467-15.

Editor: D. S. Lyles

Address correspondence to José A. Melero, jmelero@isciii.es.

A.T. and L.A. contributed equally to this article.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/JVI.00467-15.

doi:10.1128/JVI.00467-15

and later with G-specific MAbs (9, 10). Antigenic groups A and B were found to correlate with genetically distinct viral groups. Studies of hRSV evolution have focused mainly on the G glycoprotein, since G is the most divergent gene product among hRSV isolates. Recent full-genome sequence analysis has confirmed that G is most informative for studies of hRSV evolution (11).

Three types of epitopes recognized by murine MAbs have been identified in the G molecule: (i) conserved epitopes, which are present in all virus isolates; (ii) group-specific epitopes, which are shared by all viruses of the same antigenic group; and (iii) strain-specific or -variable epitopes, which are shared by a subset of viruses of the same antigenic group (12). Whereas the conserved and group-specific epitopes were mapped in the central conserved region of the G-protein ectodomain, the strain-variable epitopes clustered mainly in the C-terminal third of the G protein.

One of the main evolutionary hallmarks of hRSV G protein is that whereas nucleotide changes spread uniformly along the gene, nonsynonymous (N) changes accumulate at higher rates than synonymous (S) changes in the two variable regions, reaching dN/dS values at certain sites predictive of positive selection (12–14). The fact that these sites cluster preferentially in the C-terminal third of the G-protein primary structure, like the strain-variable epitopes, was taken as tentative evidence of immune (antibody)-driven positive selection, which was proposed as an important determinant of hRSV evolution (13, 15). This type of immune selection postulated for hRSV G protein would then be similar to the well-established antigenic drift described for the influenza virus hemagglutinin (HA) (16). In this case, new influenza virus strains are positively selected with changes in residues of the HA head which are part of epitopes recognized by neutralizing Abs. The new strains can thus reinfect the same human population despite the presence of preexisting antibodies against strains of previous epidemics.

General patterns of virus evolution are better discerned when viruses are sampled from different places over long time periods. Hence, we decided to reassess the genetic evolution of the antigenic group A of hRSV, since sequence information is most abundant for this group of viruses and since two sets of MAbs isolated in our laboratory (17, 18) could be used to compare genetic and antigenic changes in hRSV G protein. The results obtained indicate that group A viruses have diversified during their recorded history in branches (or clades) of different evolutionary significance and temporal dominance. However, epitopes recognized by strain-variable MAbs remain unchanged for long time periods, not showing signs of antigenic drift despite extensive sequence variation of the G glycoprotein.

MATERIALS AND METHODS

Clinical samples and virus isolation. Samples from Hospital Gregorio Marañón (HGM) (Madrid, Spain) were kindly provided by the HGM BioBank. Informed consent was obtained from the patients' parents or guardians. Samples, diluted in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, were used to infect HEp-2 cell monolayers growing in 24-well plates, as described previously (15). When cytopathic effect was evident, cells were scrapped off into the medium and the suspension was stored at −80°C. Samples from Instituto Conmemorativo Gorgas (Panamá, Panama) were collected as part of surveillance activities for influenza virus and other respiratory viruses, as approved by the Institute Ethical Committee.

RNA extraction, DNA amplification, and sequencing of the G-protein gene. Sequencing was done from total RNA automatically extracted from the frozen clinical specimens using QIAamp MinElute virus spin kit and the QIAcube (Qiagen), following the manufacturer's instructions. The full-length G-protein gene was amplified by SuperScript III one-Step reverse transcription-PCR (RT-PCR) (Invitrogen) and primers OG1-21 (5′-GGGGCAAATGCAACCATGTCC-3′; nucleotides [nt] 1 to 21 of the G gene; positive sense) and F164 (5′-GTTATGACACTGGTATACCAAC C-3′; nt 141 to 164 of the F gene, negative sense). PCR products were subjected to forward and reverse cycle sequencing with the BigDye Terminator 3.1 kit (Applied Biosystems) and the above-described primers.

Sequence data and BLAST search. The sequences reported here were aligned with ClustalX 1.81 (19) and manually edited with BioEdit version 7.0.9.1 (20). Other sequences were retrieved from GenBank. Since many sequences were not full length, a total of 2,167 sequences which spanned nt 312 to the end of the G-protein gene (i.e., most of the protein ectodomain) were selected and included in the study. These sequences were aligned with the online version of MAFFT v7 software (21). Duplicate sequences were identified with the ElimDupes tool (http://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html) (22) and deleted. The remaining 1,485 unique sequences used in this study and the corresponding GenBank accession numbers are listed in Table S1 in the supplemental material.

Phylogenetic analysis by Bayesian MCMC and maximum-likelihood methods. Phylogenetic analysis by the Markov chain Monte Carlo (MCMC) method was performed with the BEAST v1.7.4 package (http://beast.bio.ed.ac.uk) (23, 24), using the GTR + invariant + Gamma model selected as the best-fitting nucleotide substitution model for hRSV G-protein sequences by using hierarchical likelihood ratio testing, implemented in the ModelTest software version 3.06 (25) The data set was analyzed using the Bayesian skyline model, assuming a relaxed (uncorrelated log normal) molecular clock. MCMC chains were run to achieve convergence, which was confirmed with Tracer v1.6.0 (http://beast.bio.ed.ac.uk/Tracer). Statistical uncertainty in parameter estimates is given by the 95% highest-probability density (HPD) values. The data obtained in the MCMC analysis were also used to infer a maximum clade credibility (MCC) tree with Tree-Annotator v1.4.7 and FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). MEGA software version 6 was used for the maximum-likelihood phylogenetic analysis (26).

Fluorescent labeling of infected cells with MAbs. HEp-2 cells growing in 96-well microtiter plates were infected at a multiplicity of infection (MOI) of ~ 0.5 PFU/cell with viruses representative of the hRSV A genotypes. Twenty-four hours later, cells were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20, fixed with 80% acetone, and incubated with anti-G MAbs, followed by fluorescein-linked antibody (GE Healthcare). Cell-associated fluorescence was measured with a Tecan Infinite 200 Pro (Tecan Group Ltd.). In addition, cells were examined with a UV-illuminated Nikon Eclipse TS100 microscope.

Nucleotide sequence accession numbers. The sequences reported here were deposited in the GenBank database under accession numbers KF300969 and KF300971 to KF301019 for sequences from Panama and KP792352 to KP792376 for sequences from Madrid.

RESULTS

Evolution and dominance of group A genotypes over time. Group A viruses were classified by Peret et al. into five genotypes (27), to which nine new genotypes have been added over time by different authors without uniform criteria. It was thus considered necessary to reevaluate the current classification of group A genotypes. Hence, 1,485 unique sequences (from nucleotide 312 to the end) of the G gene were withdrawn from GenBank, together with genotype information, when available. The aligned sequences were used to assemble the maximum clade credibility (MCC) tree shown in Fig. 1A. Eleven of the 14 different genotypes previously described (GA1, GA2, GA3, GA4, GA5, GA6, GA7, NA1, NA2, NA4, and ON1) were identified in the tree. The NA3, SAA1, and SAA2 genotypes were not included, since only partial C-terminal

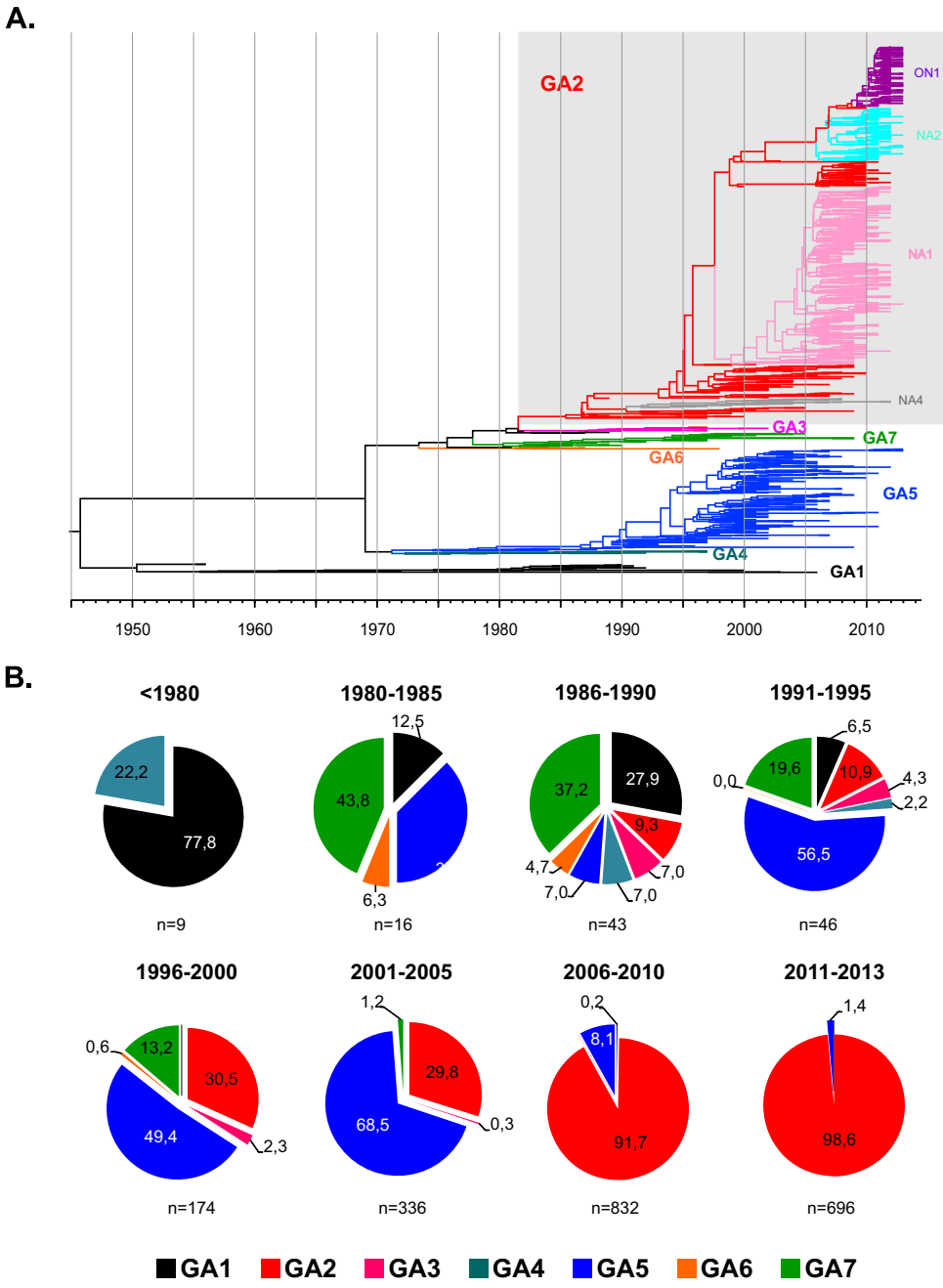


FIG 1 Phylogeny of hRSV group A viruses and genotype temporal dominance. (A) Maximum clade credibility (MCC) tree from Bayesian analysis of 1,485 unique nucleotide sequences of the G-protein gene ectodomain of hRSV group A retrieved from GenBank. Clades are colored according the genotype classification shown in Table 1. (B) Frequencies of the different genotypes in 5-year periods from 1956 to 2013. The number of sequences (*n*) included in each period is indicated below the charts.

sequences are available in the databases. The genetic *P* distances between individual genotypes, as well as within each genotype, were calculated (Table 1). The highest intragenotypic *P* distance (0.049) was found in the GA1 genotype which includes some of the oldest hRSV strains (Long and A2). This *P* value was thus taken as the minimal threshold for sorting viruses into different genotypes. Using this criterion, the NA1, NA2, NA4, and ON1 genotypes were reclassified into the GA2 genotype, leaving only seven well-recognized genotypes (GA1 to GA7) within group A of hRSV (Table 2).

The most recent common ancestor (MRCA) of all group A sequences dated back to the 1940s, when a major split into two branches occurred (Fig. 1A). One of the branches included viruses of the GA1 genotype. The other branch split in the early 1970s into two new branches. One of them gave rise to the GA4 and GA5 genotypes. Whereas only few GA4 viruses circulated for a short time period, the GA5 genotype has dominated that branch and has survived until today. The other new branch diversified with time in the group A genotypes, GA2, GA3, GA6, and GA7. It is worth emphasizing that the major branching events seen in Fig. 1A oc-

TABLE 1 Genetic distances between and within the genotypes described in the literature for 2,167 hRSV group A sequences of the G-protein ectodomain

Genotype	Genetic distance (*P* value)[a]										
	GA1	NA1	NA2	NA4	ON1	GA2	GA3	GA4	GA5	GA6	GA7
GA1	0.049										
NA1	0.165	0.018									
NA2	0.169	0.025	0.016								
NA4	0.148	0.057	0.064	0.029							
ON1	0.178	0.033	0.022	0.068	0.012						
GA2	0.158	**0.035**	**0.041**	**0.048**	**0.045**	0.037					
GA3	0.148	0.064	0.070	0.042	0.077	0.057	0.022				
GA4	0.143	0.097	0.103	0.076	0.111	0.091	0.067	0.044			
GA5	0.169	0.109	0.116	0.094	0.124	0.106	0.087	0.088	0.033		
GA6	0.140	0.084	0.090	0.064	0.096	0.076	0.059	0.071	0.090	0.025	
GA7	0.149	0.080	0.086	0.059	0.093	0.073	0.051	0.075	0.094	0.069	0.029

[a] Pairwise distances were calculated between individual genotypes, as well as within each genotype, using MEGA software version 6. BioEdit version 7.0.9.1 was used for amino acid analysis (20). Boldface indicates *P* values between genotypes GA2 and NA1, NA2, NA4, and ON1 that are below the intragenotypic distance for GA1, which was taken as the minimal threshold for clustering sequences in different genotypes. Therefore, the NA1, NA2, NA4, and ON1 genotypes were regrouped within the GA2 genotype in Table 2.

curred only occasionally, while diversification into genotypes occurred more frequently.

It is also evident from Fig. 1A that cocirculation of genotypes occurred throughout most of the known history of group A viruses, except on two occasions. One was before mid-1970s, and the other is today. This is best visualized in Fig. 1B, where the numbers of recorded sequences from different genotypes are grouped and color coded in 5-year intervals. GA1 prevailed before 1980, although the low number of samples from this time period prevents a definitive conclusion about genotype dominance. During the 1980s and 1990s, viruses from almost all genotypes were circulating, with fluctuating dominance. This situation was highlighted in numerous publications from those dates (see, for instance references 27 and 28); however, after 2000 and particularly after 2005, the proportion of viruses belonging to the GA2 genotype steadily increased, to become almost exclusive after 2010. Since the data in Fig. 1B have been extracted from multiple studies using different set of primers and slightly different methods (see Table S1 in the supplemental material), it is unlikely that sampling or geographical bias may account for the observed shift in GA2 dominance. However, very low-level circulation of viruses belonging to other genotypes cannot be excluded, as recently exemplified for group B viruses (29).

Current situation. Recent publications have drawn attention to the shift from "multiple genotype circulation to prolonged circulation of predominant genotypes," as seen in Belgium between 1996 and 2011 (30). In addition, a novel GA2 variant with a 72-nt duplication, named ON1 and first detected in Ontario (Canada) in December 2010 (31), has spread rapidly worldwide (32–34), exacerbating the current dominance of the GA2 genotype.

Access to very recent hRSV samples from Madrid (Spain) and Panama allowed us to assess the present genotype dominance in these two places, which are geographically distant and with different climates. Ectodomain G sequences of 49 samples from Panama and 25 samples from Madrid were determined and used to build the phylogenetic tree shown in Fig. 2 with representatives of the main group A genotypes. The Madrid samples were from the epidemics in 2007 to 2008, 2012 to 2013, and 2013 to 2014. The Panama sequences were from 2010, 2011, and 2012. Among the Madrid samples, the GA5 genotype represented a minority of sequences from 2007 to 2008; in contrast, most samples from this epidemic together with all sequences from the last two epidemics were clustered in the GA2 genotype. Madrid viruses from the last two epidemics contained the 72-nt duplication characteristic of the ON1 variant. Sequences from Panama were all clustered within GA2, most of them within ON1. Clearly, sequences from both places were interlocked in the tree, strengthening the idea of temporal rather than local clustering of hRSV strains and the present dominance of the GA2 genotype.

Reactivity with strain-specific anti-G monoclonal antibodies. Some of the strain-variable MAbs isolated in our laboratory have been used previously to assess the antigenic relatedness of

TABLE 2 Genetic distances (*P* values) within and between the genotypes in which group A sequences of the G-protein ectodomain of hRSV were reclassified in this study

Genotype	Genetic distance (*P* value)[a]						
	GA1	GA2	GA3	GA4	GA5	GA6	GA7
GA1	0.049						
GA2	0.166	0.031					
GA3	0.148	0.065	0.022				
GA4	0.143	0.098	0.067	0.044			
GA5	0.169	0.111	0.087	0.088	0.033		
GA6	0.140	0.084	0.059	0.071	0.090	0.025	
GA7	0.149	0.081	0.051	0.075	0.094	0.069	0.029

[a] Pairwise distances were calculated as for Table 1. Note that values for GA2 slightly differ from those in Table 1 as result of genotype reclassification.

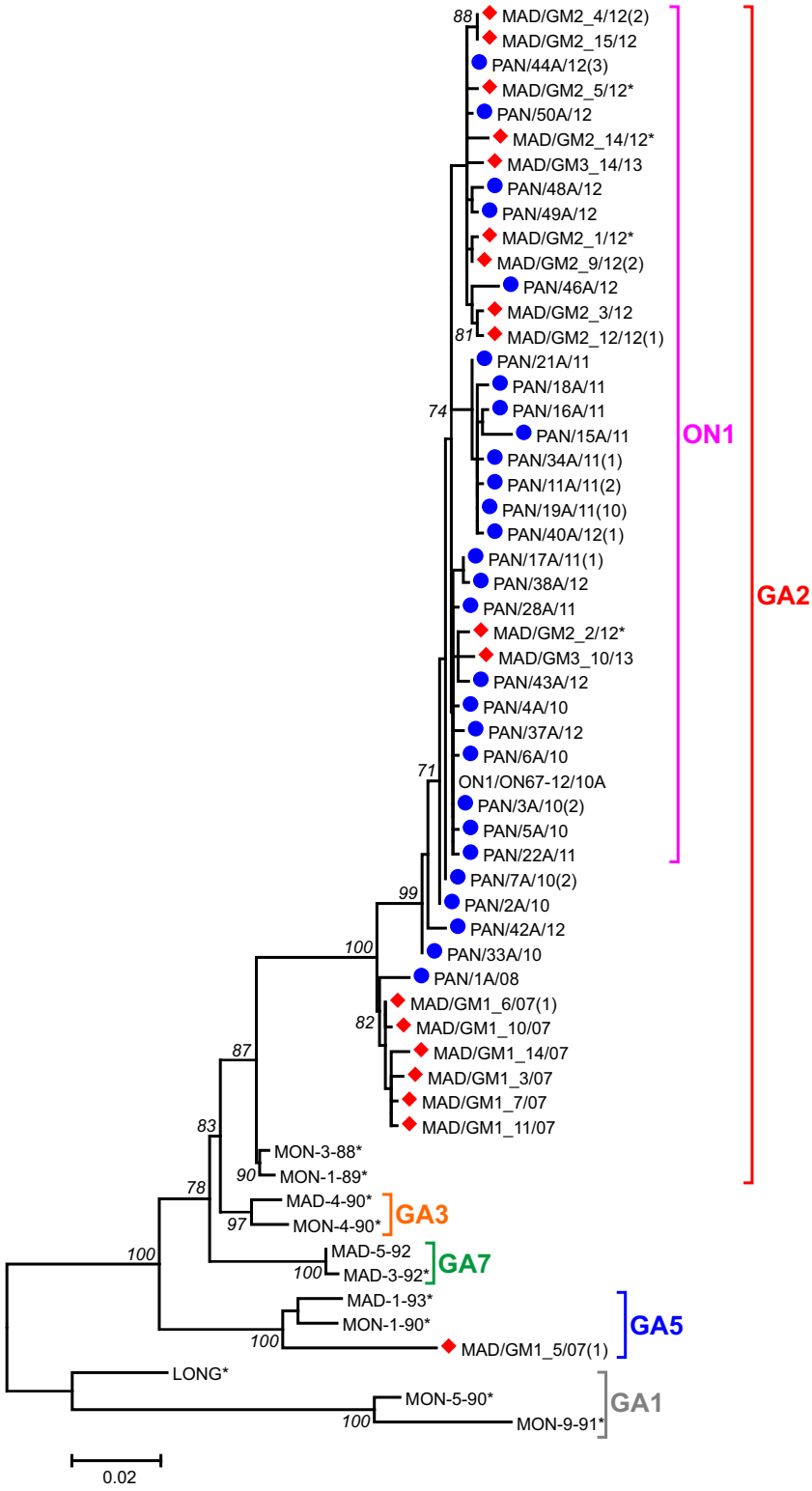
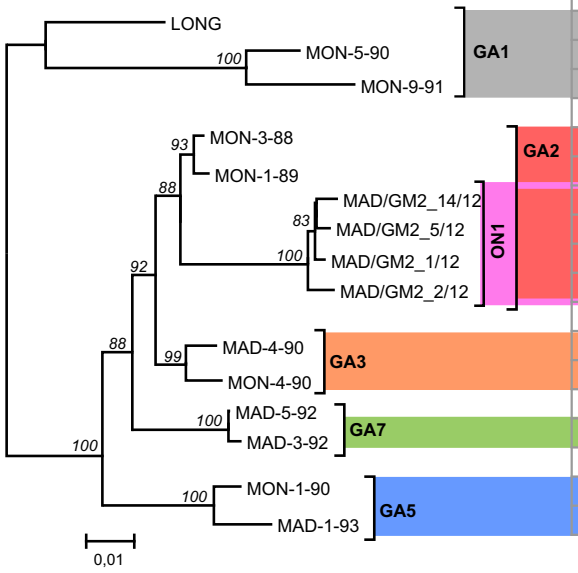


FIG 2 Phylogenetic tree of hRSV group A sequences from recent epidemics in Madrid and Panama. The maximum-likelihood phylogenetic tree was constructed on the basis of nucleotide sequences of the G-protein ectodomain obtained from Madrid (diamonds) and Panama (circles) samples. Virus nomenclature follows the general consensus, with the last two digits referring to year of isolation. The bar represents 0.02 nucleotide substitution per site, and the tree is unrooted. Numbers at the internal nodes represent the bootstrap probabilities (1,000 replicates). Only bootstrap values of >70 are shown. The number of sequences identical to those shown in the figure is indicated in parentheses at right of the sample name. Asterisks denote viruses included in the analysis shown in Fig. 3 and 4.



Strain	MAbs selected with Long				MAbs selected with Mon-3-88						
	63G	25G	78G	68G	021/12G	021/10G	021/9G	021/8G	021/7G	021/16G	021/1G
LONG	100	100	100	100	0	5	0	0	0	1	100
MON/5/90	109	67	135	135	0	5	0	0	0	0	100
MON/9/91	19	72	112	104	0	15	13	0	0	0	100
MON/3/88	96	0	0	0	100	100	100	100	100	100	100
MON/1/89	80	0	0	0	104	100	108	117	133	114	100
MAD/GM2_14/12	6	1	0	0	96	88	58	96	1	91	100
MAD/GM2_1/12	94	0	0	0	112	99	71	119	42	105	100
MAD/GM2_5/12	82	0	0	0	99	95	64	111	32	102	100
MAD/GM2_2/12	72	0	0	0	92	98	91	97	132	101	100
MAD/4/90	89	0	0	0	92	100	87	98	1	111	100
MON/4/90	15	3	1	1	74	85	42	78	72	3	100
MAD/3/92	75	0	1	1	90	88	94	92	0	84	100
MON/1/90	77	0	0	0	90	90	48	90	66	92	100
MAD/1/93	70	0	0	0	81	79	45	80	24	84	100

FIG 3 Reactivity of group A viruses with MAbs. Sequences of the viral strains used in this experiment were used to build the phylogenetic tree shown on the left, as for Fig. 2. Each virus was used to infect HEp-2 cell cultures, which were stained at 24 h after infection with the indicated MAbs and anti-mouse fluorescein-linked antibody (GE Healthcare). Two panels of strain-variable MAbs were used: one panel included MAbs 63G, 25G, 78G, and 68G, obtained from mice inoculated with the Long strain of hRSV (17), and the other included MAbs 021/12G, 021/10G, 021/9G, 021/8G, 021/7G, and 021/16G, obtained from mice inoculated with Mon/3/88 virus (18). MAb 021/1G, which recognizes a conserved epitope of hRSV G protein, was included as control. Numbers shown within the boxes are the fluorescence values after normalization, so that fluorescence of Long with each Long-specific MAb was normalized to 100% and similarly for the Mon/3/88 virus with the MAbs specific for this virus. Squares with fluorescence values of >50% have a black background, those with values between 25% and 50% have a gray background, and those with values of <25% have a white background. The results are representative of five independent determinations.

hRSV strains collected through relatively short time periods (15, 35, 36). Hence, a set of hRSV group A viruses, covering the entire recorded history of this antigenic group and representing all genotypes shown in Table 2 except GA4 and GA6 (for which viruses were not available), was used to reevaluate reactivity with the strain-variable MAbs.

The results shown in Fig. 3 show two apparent antigenic subgroups according to reactivity with the MAb panel. One subgroup included the viruses representative of the GA1 genotype, which reacted efficiently with MAbs 25G, 78G, and 68G raised against the Long strain but lacked reactivity with the strain-variable MAbs raised against the Mon/3/88 virus. The exception was MAb 63G, which, as originally reported, showed cross-reactivity with an extended set of viruses (but not all) without any obvious trend (17). The other subgroup included viruses from genotype GA2, including its ON1 variant and genotypes GA3, GA7, and GA5. All these viruses lacked reactivity with the MAbs raised against the Long strain (except the noted 63G) but reacted with most MAbs raised against Mon/3/88, with some exceptions discussed below. The results shown in Fig. 3 were in good agreement with the fluorescence patterns of infected cells stained with the MAbs (Fig. 4).

It is worth stressing that the patterns of MAb reactivity shown in Fig. 3 and 4 did not show a clear association with viral genotypes, except as noted with GA1. For instance, MAb 021/16G did not react with Mon/4/90 but reacted efficiently with Mad/4/90 from the same genotype (GA3). Exactly the opposite was true for MAb 021/7G with the same two viruses. Antibody reactivity also could not be associated with time of virus isolation. Remarkably, the epitopes of MAbs 25G, 78G, and 68G raised against the Long strain of 1956 were preserved in viruses isolated almost 40 years later in Montevideo (Fig. 3 and 4). Similarly, the epitopes recognized by MAbs raised against Mon/3/88 were preserved in most viruses isolated 24 years later in Madrid and that contained the ON1 72-nt duplication.

Figure 5A shows the alignment of sequences of the G-protein C-terminal third from viruses included in the antigenic analysis shown in Fig. 3. Residues that were changed in previously described mutants that are resistant to certain MAbs (15, 18) are indicated. When the sequence changes shown in Fig. 5A are compared with the MAb reactivities shown in Fig. 3, three main conclusions can be reached.

(i) Loss of reactivity with some MAbs coincided with certain sequence changes. For instance, MAb 63G did not react with viruses Mon/9/91, Mad/GM2_14/12, and Mon/4/90, which have the changes P206Q, K205E, and F208P, respectively, within the stretch of amino acids where epitope 63G has been mapped (37). Note, however, that the changes F208I/L in the same region did not alter reactivity with MAb 63G. Similarly, the total or partial loss of reactivity of MAbs 021/16G and 021/9G with Mon/4/90 coincided with the R244S change.

(ii) In other cases, however, residues that changed in escape mutants were totally conserved in natural isolates. For instance, residue 234, which changed in certain mutants resistant to MAb 68G, was unaltered in natural viruses. Similarly, residues 237 and 239, which changed in mutants selected with MAb 021/8G, or residue 284, which changed in mutants resistant to MAb 78G,

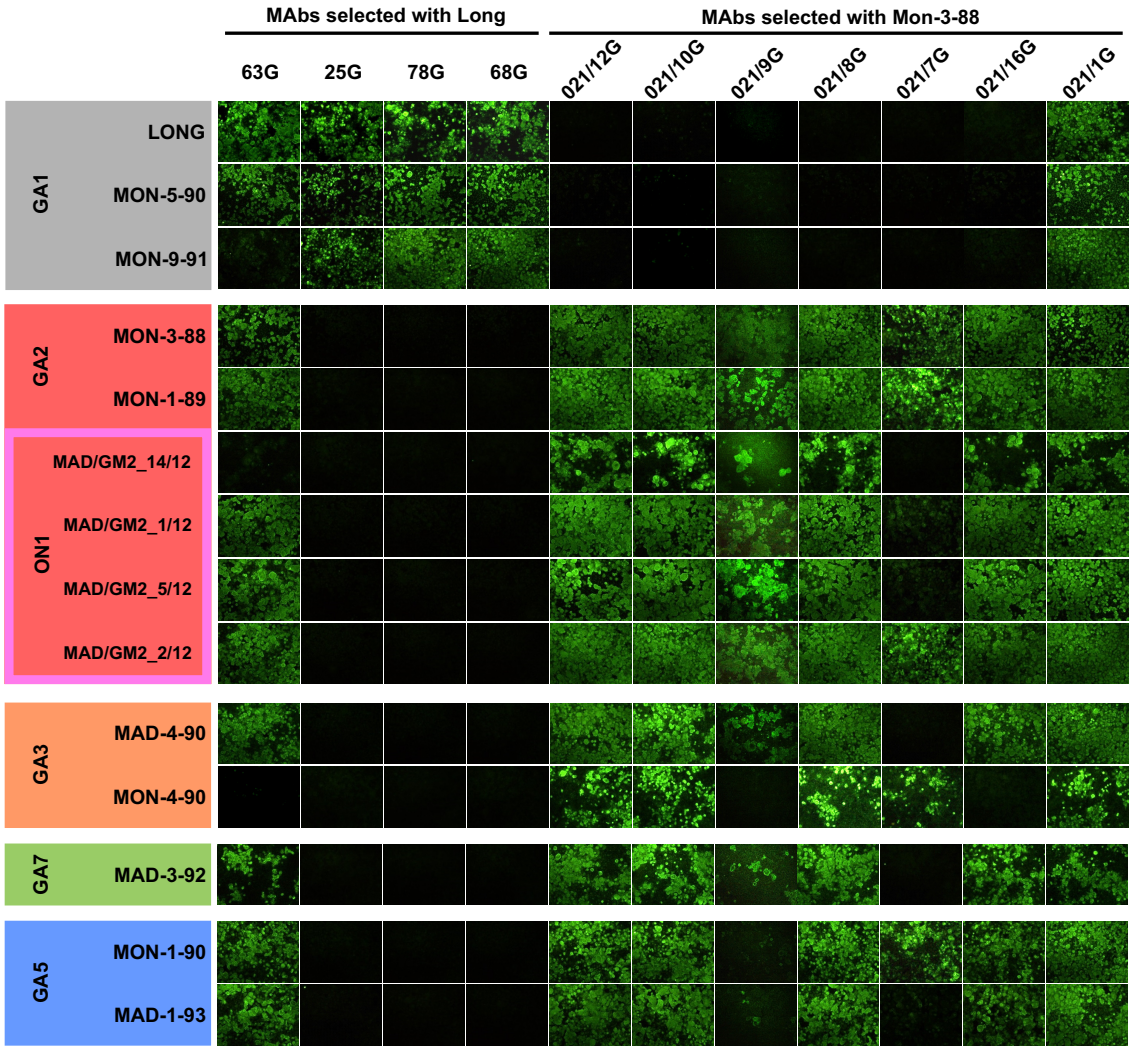


FIG 4 UV light photographs of cultures used for the quantitative analysis in Fig. 3.

were conserved in all sequences shown in Fig. 5A, irrespective of their MAb reactivity pattern.

(iii) The MAbs used for Fig. 3 have been reported to react in Western blotting with the G proteins of viruses used in their selection (7, 38). Each epitope should thus encompass several contiguous amino acids of the G-protein primary structure. It was therefore surprising to find the relatively high level of epitope conservation shown in Fig. 3 notwithstanding the extensive sequence variation of the G protein in that region (Fig. 5A). Once more, although sporadic changes were observed in individual viruses, no clear accumulation of antigenic changes with time or genetic distance was discernible.

DISCUSSION

Two enthralling findings stand out from this study: (i) the diversification of group A viruses in major branches after relatively long periods of time followed by periodic dominance of certain genotypes and (ii) the level of epitope conservation in the G glycoprotein despite the high level of sequence variation.

It is clear from Fig. 1 that diversification in major branches differs from genotype divergence not only in the magnitude of the genetic distances involved but additionally in the frequency of their respective splitting events. Hence, it is plausible that the two types of diversification have different causes, hitherto unknown. Genotype GA1, which originated from the main branching event shown in Fig. 1 is now apparently extinct. It may be that GA1 viruses exhausted the repertoire of functional amino acids that could be changed in the G glycoprotein. It is also obvious from Fig. 1B that genotype dominance has alternated in different time periods. While several genotypes cocirculated most of the time with alternating dominance, the GA2 genotype has become almost exclusive since 2005, as reported in several studies (30, 33) and observed with recent viruses from Madrid and Panama (Fig. 2). Although studied in less detail, similar shifts in genotype dominance have been reported for group B viruses (39).

What, then, are the selective forces driving branching, genotype divergence, temporal dominance, and intragenotypic evolution of hRSV? By analogy with other viruses, such as influenza A virus, antibody-driven positive selection has been proposed as a major determinant of hRSV evolution (13) to enable reinfections of the same population, an epidemiological hallmark of hRSV (40). Positive selection is supported by the high rate of dN/dS

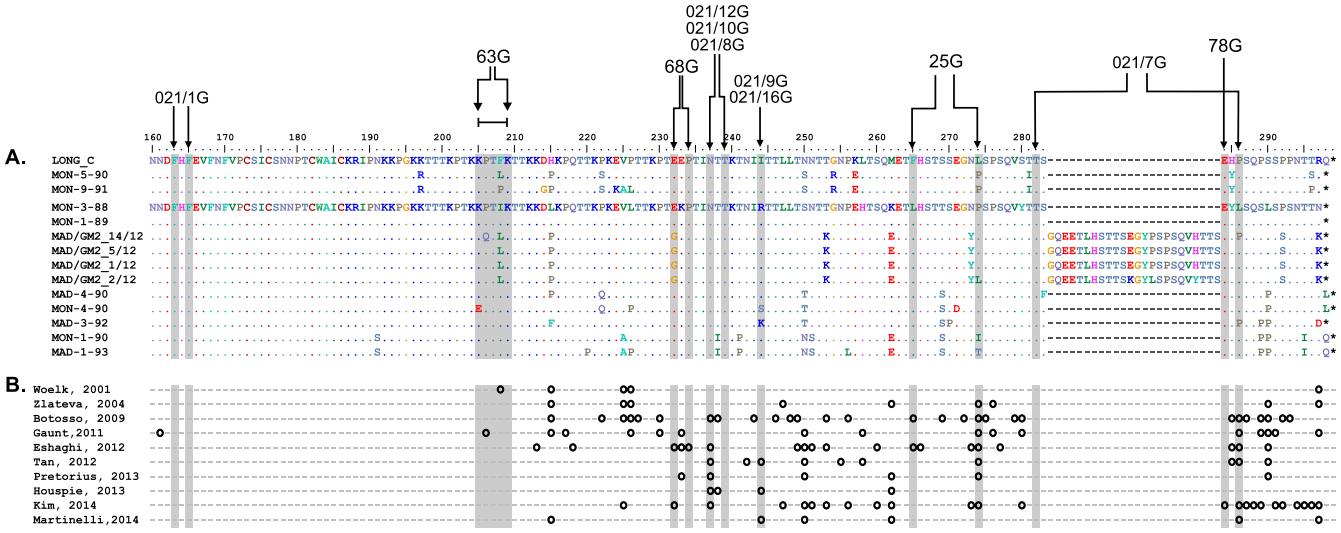


FIG 5 Sequence alignment of the C-terminal thirds of G-protein sequences. (A) Alignment of partial (C-terminal) G-protein sequences of the viruses used for Fig. 3. Numbering is shown above the Long sequence, which is used as a reference for the next two viruses. The entire sequence of Mon/3/88 is also shown as a reference for the rest of viruses. Only the amino acid changes are indicated. A lack of change is denoted by a dot. Asterisks indicate stop codons. Note the 72-nt duplicated sequence in four viruses, which forces the gaps denoted by hyphens in the other sequences. Residues that showed changes in mutants selected with the indicated MAbs are indicated by arrows at the top. (B) Sites of positive selection predicted in the indicated studies (11, 13, 30, 31, 33, 49–53) are denoted by small circles below the corresponding amino acid.

substitutions and by predictions of positively selected changes at certain sites of the G glycoprotein. Figure 5B shows sites of positive selection in the C-terminal third of the G glycoprotein predicted in different studies. The accumulation of those sites (but not all) in the same region of the G protein where strain-variable epitopes are clustered has been a major argument for the antibody-driven positive selection hypothesis of hRSV evolution.

Often, however, changes in sites of positive selection do not correlate with changes in MAb reactivity. For instance, one of the most recurrently predicted sites of positive selection is residue 237, where MAb 021/8G selected an escape mutant with the change N237Y (18). Paradoxically, however, amino acid 237 is conserved in all sequences shown in Fig. 5A, including those of viruses of the GA1 genotype which are not recognized by MAb 021/8G. In other sites of positive selection, such as residue 274, changes found in certain viruses (L to P, I, or T) do not correlate with the MAb reactivity shown in Fig. 3. Hence, no definitive association between altered antibody reactivity and sites of predicted positively selected changes could be shown.

Additionally, when Fig. 3 and 5 are globally examined, no obvious correspondence between genotype genetic relatedness and MAb reactivity pattern is observed. These results are generally in agreement with previous reports that detected sporadic antigenic changes in viruses circulating in Argentina and Chile (35, 41) or in Germany (36) with the MAbs used in this study but without any distinctive trend.

It may be argued that murine MAbs may not represent the repertoire of human antibodies raised after hRSV infection. Without excluding this possibility, it is worth mentioning that linear epitopes recognized by antibodies present in human sera have been detected in peptides (42) or protein segments from the G glycoprotein C-terminal third (8).

It should be also stressed that most murine MAbs raised against the G glycoprotein are weak neutralizers (17, 43). Furthermore, most of the neutralizing activity found in human immunoglobulins is directed against highly conserved epitopes of the F glycoprotein (44, 45). In other words, the immune pressure afforded by anti-G antibodies is expected to be only marginal, if any. Hence, it may be that hRSV reinfections are determined by short-lived (or weak) antibody responses rather than selection of antigenic variants. Indeed, recent studies have provided evidence that reinfections in children are caused almost as frequently by heterologous viruses as by viruses of the homologous antigenic group (46, 47). In addition, although partial group-specific neutralizing responses were noted in very young children after hRSV infections, neutralization was reported to be equally effective against contemporary or historical viruses, suggesting no significant antigenic drift (48). Indeed, similar results have been obtained in our laboratory with a limited set of infant sera, in which neither neutralizing nor G-protein binding antibodies were strain dependent within the same antigenic group as the infecting virus (A. Trento et al., unpublished data).

In summary, this study highlights a complex pattern of group A hRSV diversification, with major branching and temporal genotype dominance over time that could not be directly related to antigenic changes. Although some weak antibody selection cannot be excluded, our results do not support the idea that the high level of sequence variation in the G glycoprotein is the result of an antigenic drift similar to that of influenza A virus HA (16). It is thus likely that other factors contribute to the accumulation of sequence changes in hRSV G protein. The high plasticity of this protein to incorporate drastic sequence changes without apparent alterations in virus fitness should be stressed (12). Is it possible, then, that the seemingly positive selection of changes in RSV G protein is the result of a high mutation rate (as generally in RNA viruses) together with selective constrains other than immune selection? For instance, is it possible that bottleneck effects occurring during virus transmission together with a very malleable mol-

ecule may result in an apparent positive selection in hRSV G protein? It may be that hRSV has found an "entropically" favorable solution for the G protein so that its unusual amino acid sequence and its added malleability maintain functionality together with an apparent positive selection. Further studies with well-selected hRSV strains should help to discern among this possibility and others lying behind the intriguing paradoxes of hRSV evolution.

ACKNOWLEDGMENTS

We thank Juan Ortín, Isidoro Martínez, and Jason McLellan for critical readings of the manuscript and very useful comments and members of the Genomic Core Facility at the Instituto de Salud Carlos III for technical help. We particularly acknowledge the patients in this study for their participation and the HGM BioBank integrated in RETICS, National Network Biobanks, and collaborating centers for the generous gifts of clinical samples used in this work.

This work was supported by grant SAF2012-31217 from Plan Nacional I+D+I (J.A.M., Ministerio de Economía y Competitividad, Spain) and grant CAP10-001, Senacyt-Panamá. The HGM BioBank, integrated in National Network Biobanks, is supported by Instituto de Salud Carlos III, Spanish Science and Innovation Ministry (grant number PT13/0010/0028) and Fundación para la Investigación y Prevención del SIDA (FIPSE), Spain.

REFERENCES

1. **Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA, Singleton RJ, O'Brien KL, Roca A, Wright PF, Bruce N, Chandran A, Theodoratou E, Sutanto A, Sedyaningsih ER, Ngama M, Munywoki PK, Kartasasmita C, Simoes EA, Rudan I, Weber MW, Campbell H.** 2010. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. Lancet **375:**1545–1555. http://dx.doi.org/10.1016/S0140-6736(10)60206-1.
2. **Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE.** 2005. Respiratory syncytial virus infection in elderly and high-risk adults. N Engl J Med **352:**1749–1759. http://dx.doi.org/10.1056/NEJMoa043951.
3. **Collins PL, Melero JA.** 2011. Progress in understanding and controlling respiratory syncytial virus: still crazy after all these years. Virus Res **162:**80–99. http://dx.doi.org/10.1016/j.virusres.2011.09.020.
4. **Levine S, Klaiber-Franco R, Paradiso PR.** 1987. Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. J Gen Virol **68:**2521–2524. http://dx.doi.org/10.1099/0022-1317-68-9-2521.
5. **Walsh EE, Hruska J.** 1983. Monoclonal antibodies to respiratory syncytial virus proteins: identification of the fusion protein. J Virol **47:**171–177.
6. **Gruber C, Levine S.** 1985. Respiratory syncytial virus polypeptides. V. The kinetics of glycoprotein synthesis. J Gen Virol **66:**1241–1247.
7. **Garcia-Beato R, Martinez I, Franci C, Real FX, Garcia-Barreno B, Melero JA.** 1996. Host cell effect upon glycosylation and antigenicity of human respiratory syncytial virus G glycoprotein. Virology **221:**301–309. http://dx.doi.org/10.1006/viro.1996.0379.
8. **Palomo C, Cane PA, Melero JA.** 2000. Evaluation of the antibody specificities of human convalescent-phase sera against the attachment (G) protein of human respiratory syncytial virus: influence of strain variation and carbohydrate side chains. J Med Virol **60:**468–474. http://dx.doi.org/10.1002/(SICI)1096-9071(200004)60:4<468::AID-JMV16>3.0.CO;2-E.
9. **Anderson LJ, Hierholzer JC, Tsou C, Hendry RM, Fernie BF, Stone Y, McIntosh K.** 1985. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J Infect Dis **151:**623–633.
10. **Mufson MA, Orvell C, Rafnar B, Norrby E.** 1985. Two distinct subtypes of human respiratory syncytial virus. J Gen Virol **66:**2111–2124. http://dx.doi.org/10.1099/0022-1317-66-10-2111.
11. **Tan L, Lemey P, Houspie L, Viveen C, Jansen NJ, van Loon AM, Wiertz E, van Bleek GM, Martin DP, Coenjaerts FE.** 2012. Genetic variability among complete human respiratory syncytial virus subgroup A genomes: bridging molecular evolutionary dynamics and epidemiology. PLoS One **7:**e51439. http://dx.doi.org/10.1371/journal.pone.0051439.
12. **Melero JA, Garcia-Barreno B, Martinez I, Pringle CR, Cane PA.** 1997. Antigenic structure, evolution and immunobiology of human respiratory syncytial virus attachment (G) protein. J Gen Virol **78:**2411–2418.
13. **Woelk CH, Holmes EC.** 2001. Variable immune-driven natural selection in the attachment (G) glycoprotein of respiratory syncytial virus (RSV). J Mol Evol **52:**182–192.
14. **Tan L, Coenjaerts FE, Houspie L, Viveen MC, van Bleek GM, Wiertz EJ, Martin DP, Lemey P.** 2013. The comparative genomics of human respiratory syncytial virus subgroups A and B: genetic variability and molecular evolutionary dynamics. J Virol **87:**8213–8226. http://dx.doi.org/10.1128/JVI.03278-12.
15. **Garcia O, Martin M, Dopazo J, Arbiza J, Frabasile S, Russi J, Hortal M, Perez-Brena P, Martinez I, Garcia-Barreno B.** 1994. Evolutionary pattern of human respiratory syncytial virus (subgroup A): cocirculating lineages and correlation of genetic and antigenic changes in the G glycoprotein. J Virol **68:**5448–5459.
16. **Knossow M, Skehel JJ.** 2006. Variation and infectivity neutralization in influenza. Immunology **119:**1–7. http://dx.doi.org/10.1111/j.1365-2567.2006.02421.x.
17. **Garcia-Barreno B, Palomo C, Penas C, Delgado T, Perez-Brena P, Melero JA.** 1989. Marked differences in the antigenic structure of human respiratory syncytial virus F and G glycoproteins. J Virol **63:**925–932.
18. **Martinez I, Dopazo J, Melero JA.** 1997. Antigenic structure of the human respiratory syncytial virus G glycoprotein and relevance of hypermutation events for the generation of antigenic variants. J Gen Virol **78:**2419–2429.
19. **Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG.** 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **25:**4876–4882. http://dx.doi.org/10.1093/nar/25.24.4876.
20. **Hall TA.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 93/98/NT41. Nucleic Acids Symp Ser **41:**95–98.
21. **Katoh K, Standley DM.** 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol. Evol **30:**772–780. http://dx.doi.org/10.1093/molbev/mst010.
22. **Kuiken C, Yusim K, Boykin L, Richardson R.** 2005. The Los Alamos hepatitis C sequence database. Bioinformatics **21:**379–384. http://dx.doi.org/10.1093/bioinformatics/bth485.
23. **Drummond AJ, Rambaut A, Shapiro B, Pybus OG.** 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. Mol Biol Evol **22:**1185–1192. http://dx.doi.org/10.1093/molbev/msi103.
24. **Drummond AJ, Rambaut A.** 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol **7:**214. http://dx.doi.org/10.1186/1471-2148-7-214.
25. **Posada D, Crandall KA.** 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics **14:**817–818. http://dx.doi.org/10.1093/bioinformatics/14.9.817.
26. **Tamura K, Stecher G, Peterson D, Filipski A, Kumar S.** 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol **30:**2725–2729. http://dx.doi.org/10.1093/molbev/mst197.
27. **Peret TC, Hall CB, Schnabel KC, Golub JA, Anderson LJ.** 1998. Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. J Gen Virol **79:**2221–2229.
28. **Cane PA, Matthews DA, Pringle CR.** 1994. Analysis of respiratory syncytial virus strain variation in successive epidemics in one city. J Clin Microbiol **32:**1–4.
29. **Agoti CN, Gitahi CW, Medley GF, Cane PA, Nokes DJ.** 2013. Identification of group B respiratory syncytial viruses that lack the 60-nucleotide duplication after six consecutive epidemics of total BA dominance at coastal Kenya. Influenza Other Respir Viruses **7:**1008–1012. http://dx.doi.org/10.1111/irv.12131.
30. **Houspie L, Lemey P, Keyaerts E, Reijmen E, Vergote V, Vankeerberghen A, Vaeyens F, De BH, Van RM.** 2013. Circulation of HRSV in Belgium: from multiple genotype circulation to prolonged circulation of predominant genotypes. PLoS One **8:**e60416. http://dx.doi.org/10.1371/journal.pone.0060416.
31. **Eshaghi A, Duvvuri VR, Lai R, Nadarajah JT, Li A, Patel SN, Low DE, Gubbay JB.** 2012. Genetic variability of human respiratory syncytial virus A strains circulating in Ontario: a novel genotype with a 72 nucleotide G gene duplication. PLoS One **7:**e32807. http://dx.doi.org/10.1371/journal.pone.0032807.
32. **Prifert C, Streng A, Krempl CD, Liese J, Weissbrich B.** 2013. Novel

respiratory syncytial virus a genotype, Germany, 2011-2012. Emerg Infect Dis **19:**1029–1030. http://dx.doi.org/10.3201/eid1906.121582.
33. **Martinelli M, Frati ER, Zappa A, Ebranati E, Bianchi S, Pariani E, Amendola A, Zehender G, Tanzi E.** 2014. Phylogeny and population dynamics of respiratory syncytial virus (Rsv) A and B. Virus Res **189:**293–302. http://dx.doi.org/10.1016/j.virusres.2014.06.006.
34. **Agoti CN, Otieno JR, Gitahi CW, Cane PA, Nokes DJ.** 2014. Rapid spread and diversification of respiratory syncytial virus genotype ON1, Kenya. Emerg Infect Dis **20:**950–959. http://dx.doi.org/10.3201/eid2006.131438.
35. **Galiano MC, Luchsinger V, Videla CM, De Souza L, Puch SS, Palomo C, Ricarte C, Ebekian B, Avendano L, Carballal G.** 2005. Intragroup antigenic diversity of human respiratory syncytial virus (group A) isolated in Argentina and Chile. J Med Virol **77:**311–316. http://dx.doi.org/10.1002/jmv.20456.
36. **Adams O, Werzmirzowsky J, Hengel H.** 2013. Genetic analysis and antigenic characterization of human respiratory syncytial virus group A viruses isolated in Germany 1996-2008. Virus Genes **47:**210–218. http://dx.doi.org/10.1007/s11262-013-0936-9.
37. **Garcia-Barreno B, Delgado T, Akerlind-Stopner B, Norrby E, Melero JA.** 1992. Location of the epitope recognized by monoclonal antibody 63G on the primary structure of human respiratory syncytial virus G glycoprotein and the ability of synthetic peptides containing this epitope to induce neutralizing antibodies. J Gen Virol **73:**2625–2630. http://dx.doi.org/10.1099/0022-1317-73-10-2625.
38. **Palomo C, Garcia-Barreno B, Penas C, Melero JA.** 1991. The G protein of human respiratory syncytial virus: significance of carbohydrate side-chains and the C-terminal end to its antigenicity. J Gen Virol **72:**669–675. http://dx.doi.org/10.1099/0022-1317-72-3-669.
39. **Trento A, Casas I, Calderon A, Garcia-Garcia ML, Calvo C, Perez-Brena P, Melero JA.** 2010. Ten years of global evolution of the human respiratory syncytial virus BA genotype with a 60-nucleotide duplication in the G protein gene. J Virol **84:**7500–7512. http://dx.doi.org/10.1128/JVI.00345-10.
40. **Henderson FW, Collier AM, Clyde WA, Jr, Denny FW.** 1979. Respiratory-syncytial-virus infections, reinfections and immunity. A prospective, longitudinal study in young children. N Engl J Med **300:**530–534.
41. **Galiano MC, Palomo C, Videla CM, Arbiza J, Melero JA, Carballal G.** 2005. Genetic and antigenic variability of human respiratory syncytial virus (groups a and b) isolated over seven consecutive seasons in Argentina (1995 to 2001). J Clin Microbiol **43:**2266–2273. http://dx.doi.org/10.1128/JCM.43.5.2266-2273.2005.
42. **Cane PA.** 1997. Analysis of linear epitopes recognised by the primary human antibody response to a variable region of the attachment (G) protein of respiratory syncytial virus. J Med Virol **51:**297–304. http://dx.doi.org/10.1002/(SICI)1096-9071(199704)51:4<297::AID-JMV7>3.0.CO;2-0.
43. **Anderson LJ, Bingham P, Hierholzer JC.** 1988. Neutralization of respiratory syncytial virus by individual and mixtures of F and G protein monoclonal antibodies. J Virol **62:**4232–4238.
44. **Magro M, Mas V, Chappell K, Vazquez M, Cano O, Luque D, Terron MC, Melero JA, Palomo C.** 2012. Neutralizing antibodies against the preactive form of respiratory syncytial virus fusion protein offer unique possibilities for clinical intervention. Proc Natl Acad Sci U S A **109:**3089–3094. http://dx.doi.org/10.1073/pnas.1115941109.
45. **McLellan JS, Chen M, Leung S, Graepel KW, Du X, Yang Y, Zhou T, Baxa U, Yasuda E, Beaumont T, Kumar A, Modjarrad K, Zheng Z, Zhao M, Xia N, Kwong PD, Graham BS.** 2013. Structure of RSV fusion glycoprotein trimer bound to a prefusion-specific neutralizing antibody. Science **340:**1113–1117. http://dx.doi.org/10.1126/science.1234914.
46. **Yui I, Fujino M, Sawada A, Nakayama T.** 2014. Novel clinical features of recurrent human respiratory syncytial virus infections. J Med Virol **86:**1629–1638. http://dx.doi.org/10.1002/jmv.23809.
47. **Agoti CN, Mwihuri AG, Sande CJ, Onyango CO, Medley GF, Cane PA, Nokes DJ.** 2012. Genetic relatedness of infecting and reinfecting respiratory syncytial virus strains identified in a birth cohort from rural kenya. J Infect Dis **206:**1532–1541. http://dx.doi.org/10.1093/infdis/jis570.
48. **Sande CJ, Mutunga MN, Medley GF, Cane PA, Nokes DJ.** 2013. Group- and genotype-specific neutralizing antibody responses against respiratory syncytial virus in infants and young children with severe pneumonia. J Infect Dis **207:**489–492. http://dx.doi.org/10.1093/infdis/jis700.
49. **Zlateva KT, Lemey P, Vandamme AM, Van RM.** 2004. Molecular evolution and circulation patterns of human respiratory syncytial virus subgroup a: positively selected sites in the attachment G glycoprotein. J Virol **78:**4675–4683. http://dx.doi.org/10.1128/JVI.78.9.4675-4683.2004.
50. **Gaunt ER, Jansen RR, Poovorawan Y, Templeton KE, Toms GL, Simmonds P.** 2011. Molecular epidemiology and evolution of human respiratory syncytial virus and human metapneumovirus. PLoS One **6:**e17427. http://dx.doi.org/10.1371/journal.pone.0017427.
51. **Pretorius MA, van NS, Tempia S, Moyes J, Cohen C, Madhi SA, Venter M.** 2013. Replacement and positive evolution of subtype A and B respiratory syncytial virus G-protein genotypes from 1997-2012 in South Africa. J Infect Dis **208**(Suppl 3):S227–S237. http://dx.doi.org/10.1093/infdis/jit477.
52. **Kim YJ, Kim DW, Lee WJ, Yun MR, Lee HY, Lee HS, Jung HD, Kim K.** 2014. Rapid replacement of human respiratory syncytial virus A with the ON1 genotype having 72 nucleotide duplication in G gene. Infect Genet Evol **26:**103–112. http://dx.doi.org/10.1016/j.meegid.2014.05.007.
53. **Botosso VF, Zanotto PM, Ueda M, Arruda E, Gilio AE, Vieira SE, Stewien KE, Peret TC, Jamal LF, Pardini MI, Pinho JR, Massad E, Sant'anna OA, Holmes EC, Durigon EL.** 2009. Positive selection results in frequent reversible amino acid replacements in the G protein gene of human respiratory syncytial virus. PLoS Pathog **5:**e1000254. http://dx.doi.org/10.1371/journal.ppat.1000254.

CAPÍTULO II

CAPÍTULO II

CARACTERIZACIÓN GENÉTICA DE CEPAS DEL VRSH DEL GRUPO B, IDENTIFICADAS EN PANAMA ENTRE 2008-2012.

MANUSCRITO: GENETIC VARIABILITY OF HUMAN RESPIRATORY SYNCYTIAL VIRUS GROUP B IN PANAMA REVEALS A NOVEL GENOTYPE BA14.

Leyda E. Ábrego[1], Adriana Delfraro[4], Danilo Franco[1,3], Juan Castillo[2], Marlene Castillo[1], Brechla Moreno[1], Sandra López-Vergès[1], Juan M. Pascale[2,3] and Juan Arbiza[4]

Este estudio muestra los genotipos circulantes de VRSH-B en niños menores de 5 años de Panamá, durante los años 2008-2012. Nuestros resultados, no sólo concordaron con el predominio del genotipo BA reportado a nivel mundial, también mostraron la circulación de un genotipo nuevo "BA14". Además se presenta un análisis de sustitución de amino ácidos mostrando cambios previamente reportados y otros sólo encontrados en las cepas de Panamá.

---

[1]Deparment of Research in Virology and Biotechnology, Gorgas Memorial Institute of Health Studies, Panama City, Panama; [2]Department of Genomic and Proteomic, Gorgas Memorial Institute of Health Studies, Panama City, Panama; [3]School of Medicine, University of Panama, Panama City, Panama; [4]Virology Section, School of Sciences, University of the Republic, Montevideo, Uruguay.

Correspondence to: Juan Arbiza, Sección de Virología, Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo 11400, Uruguay. E-mail: jarbiza@fcien.edu.uy

Received: 25 November 2016 | Revised: 24 March 2017 | Accepted: 17 April 2017
DOI: 10.1002/jmv.24838

RESEARCH ARTICLE

WILEY JOURNAL OF MEDICAL VIROLOGY

Genetic variability of human respiratory syncytial virus group B in Panama reveals a novel genotype BA14

Leyda E. Ábrego[1] | Adriana Delfraro[2] | Danilo Franco[1,3] | Juan Castillo[4] | Marlene Castillo[1] | Brechla Moreno[1] | Sandra López-Vergès[1] | Juan M. Pascale[3,4] | Juan Arbiza[2]

[1] Deparment of Research in Virology and Biotechnology, Gorgas Memorial Institute of Health Studies, Panama City, Panama

[2] Virology Section, School of Sciences, University of the Republic, Montevideo, Uruguay

[3] School of Medicine, University of Panama, Panama City, Panama

[4] Department of Genomic and Proteomic, Gorgas Memorial Institute of Health Studies, Panama City, Panama

Correspondence
Juan Arbiza, Sección de Virología, Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo 11400, Uruguay.
Email: jarbiza@fcien.edu.uy

Funding information
Secretaría Nacional de Ciencias, Tecnología e Innovación Panamá (SENACYT), Grant number: CAP10-001; Agencia Nacional de Investigación e Innovación de Uruguay, (ANII); Programa de Desarrollo de las Ciencias Básicas del Uruguay, (PEDECIBA)

In Panama, human respiratory syncytial virus (HRSV) is responsible of 20-40% of acute respiratory infections in children under 5 years old. Currently, little is known about the genetic variability of HRSV in Central America and the Caribbean. Recently, we reported the genetic variability of HRSV-A, however; no studies on HRSV-B in Panama have been described yet. In this study, 24 sequences of Panamanian HRSV-B, from children (<5 years) with acute respiratory infections (ARI), collected from July 2008 to November 2012 were analyzed. All sequences share the characteristic 60-nt duplication of the BA strains. Six Panamanian strains grouped with the BA10 genotype and 12 samples clustered together in a separate monophyletic clade with an aLRT support value of 0.92 and an intra-group p-distance less than 0.07. This fulfills the criteria to consider a new genotype in HRSV, which we named BA14 genotype. Another six strains remain unclassified, but closely related to BA9, BA11, or the new BA14 genotypes, according to their genetic p-distance. Different amino acid substitutions in the Panamanian HRSV-B strains were observed, some previously described and others found only on Panamanian strains. This study contributes to the knowledge of the genetic variability and evolution of HRSV in Central America.

KEYWORDS
acute respiratory infections (ARI), genotype, group B, human respiratory syncytial virus

1 | INTRODUCTION

Human respiratory syncytial virus (HRSV) was described in 1957 in infants with bronchiolitis and since then it became a major pathogen of acute respiratory infections (ARI), mainly of lower respiratory tract infections (ALRI), in infants and young children worldwide.[1] Exposure to HRSV induces limited immune protection without long lasting memory and for this reason HRSV causes frequent re-infections.[2] It is estimated that HRSV can cause worldwide over thirty million ALRI each year, resulting in three million hospitalizations per year in children under 5 years of age. In developing countries, ALRI associated to HRSV infection is responsible for 99% of the death of children younger than 5 years.[1] HRSV epidemics occur annually during winter months in temperate climates, whereas outbreaks are associated with the rainy season in tropical climates. The climate in Panama is similar to many tropical Central American countries. The rainy season last between mid-April to -December with high humidity percentages, the rest of the year is considered dry season and the daytime humidity is lower. Studies in Panama about epidemiology of respiratory infections, showed a definite season pattern for RSV infection. The first peak of illness is observed in May and increases with the intensification of rainfall showing a major peak between August and November.[3]

HRSV is member of the *Pneumovirus* genus within the *Paramyxoviridae* family. It is an enveloped, non-segmented, negative-sense, single-stranded RNA virus of approximately 15.2 kb that encodes for 11 proteins.[4] Among these proteins, three are surface glycoproteins, namely small hydrophobic (SH), fusion (F), and attachment (G). The G protein is responsible for binding of the virus to the cell surface receptor.[5] The F glycoprotein mediates fusion of the viral and cell membranes, allowing the entrance of the virus' nucleocapsid into the cell cytoplasm and the initiation of a new infectious cycle.[6] The F and G proteins are the main targets of protective antibodies.[5,6] The F protein is relatively highly conserved, while the G protein has hypervariable regions.[7] The G protein is a type II glycoprotein that has an ectodomain including two heavily glycosylated hypervariable domains (HVR1 and HVR2), which are separated by a conserved central region.[5,7,8]

HRSV has been divided into two antigenic groups: HRSV-A and HRSV-B.[9] For genotyping HRSV, the second hypervariable region of the gene coding the G protein is commonly used due to its high degree of divergence, given that this region can reflect the overall gene variability.[8,10] To date, molecular epidemiological studies of this region have reported several genotypes for each HRSV group: 15 for HRSV-A (GA1 to GA7, SAA1, SAA2, NA1-NA4, CB-A, and a novel genotype ON1 subtype with a 72 nucleotide G gene duplication),[8,11–18] and 26 for HRSV-B (GB1 to GB5, SAB1 to SAB4, URU1, URU2, CB-B, CB-1, and BA1 to BA13).[8,12,14,16,19–25] Generally, group A strains are predominant over group B strains in epidemic years, however, both may co-circulate within the same season and same community, with one or two dominant genotypes being replaced in successive years.[8,11,23] A duplication of 60 nucleotides in the C-terminal portion of the HRSV-B G gene was first observed in strains from Buenos Aires, Argentina. This novel genotype, named BA circulated in 1999 and shared a common ancestor that was dated from 1998.[19,23] According to global epidemiological studies the BA genotype had rapidly spread to different countries worldwide, resulting in the predominant genotype of HRSV-B.[17,23,25,26]

Preliminary studies have shown the importance of HRSV in ARI in Central America, this virus has been responsible for 6.9-33% of ARI in children and 5-6% in adults.[27–29] Despite this, there are few reports on molecular epidemiology of this relevant virus in the Central American and Caribbean regions.[30,31]

Panama has a program of influenza surveillance since 1976 and the Gorgas Memorial Institute of Health Studies (GMI) was certified by the World Health Organization (WHO) as National Influenza Center (NIC) since 2007. The reports from 2011 to 2014 of the Panamanian Influenza and other Respiratory Viruses Surveillance Program showed that the frequency of HRSV in nasopharyngeal samples from children less than 5 years old with ARI ranged between 20% and 40%. Recently, genetic and antigenic characterization of Panamanian HRSV-A was reported,[18] however, there is no published information about HRSV-B genotypes circulating in the country. The purpose of this study is to analyze the genetic diversity of HRSV group B in Panama. To do this, we conducted the genetic characterization of the C-terminal third of the G genes of HRSV-B strains from samples obtained from children younger than 5 years old, collected from July 2008 to November 2012.

2 | MATERIALS AND METHODS

2.1 | Clinical samples and patients

The National influenza and other respiratory viruses surveillance program was centralized at GMI and the GMI received nasopharyngeal samples from 18 sentinel sites throughout the country. The samples were from adults and children however, 90% were from children under 5 years old. Surveillance of respiratory viruses (influenza A and B, HRSV, human metapneumovirus, human rhinovirus, adenovirus, and parainfluenza virus types 1, 2, and 3) was performed by molecular biology methods (PCR or RT-PCR genome amplification) on nasopharyngeal swabs from ambulatory and hospitalized patients with ARI. The protocols used for the diagnosis of influenza virus and other respiratory virus is provided by Centers of Disease Control (CDC) in Atlanta, GA. Positive samples for HRSV from children under 5 years of age with ALRI, collected during July 2008 to November 2012, were selected randomly and analyzed. The protocol of the study was submitted and approved by the Gorgas Memorial Institute Bioethics Committee (no. 084/CBI/ICGES/12 and no. 617/CBI/ICGES/13).

2.2 | RNA extraction and RT-PCR amplification

Total RNA was extracted from 140 μL of nasopharyngeal swabs using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. A RT-PCR followed by a nested PCR was performed; giving an amplicon of 1115 bp corresponding to the G gene and a small portion of the F gene of HRSV. RT-PCR was performed using the QIAGEN One-Step RT-PCR Kit (QIAGEN) with 5 μL of the samples' RNA and the following published primers: LG5+(5′-GGATCCCGGGGCAAATGCAAACATGTCC-3') and F164-(5'GTTATGACACTGGTATACCAACC-3).[32] Cycling conditions were 45 min at 48°C, 15 min at 95°C, 45 cycles at 95°C for 1 min, 54°C for 1 min, 68°C for 2 min, followed by 68°C for 5 min. For the nested PCR, a Taq-PCR Master Mix Kit (QIAGEN) was prepared with the following primers: LG1+ (5′-GGGGCAAATGCAAA-3′) and LGF- (5′-TTTGCTAACTGCACTGCATGT-3′) with 2 μL of the first product. The cycling conditions for the second PCR were 5 min at 95°C, 35 cycles at 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, followed by 72°C for 5 min. The products were resolved by electrophoresis in a 1.5% agarose gel stained with ethidium bromide and visualized under UV light.

2.3 | DNA sequencing

The amplified products were purified using the QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's instructions. Sequencing of both strands was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) with the same primers used in the nested PCR. The BigDye® XTerminator™ Purification Kit was used for purification of the sequence reactions and then sequenced in a 3130xl genetic analyzer (Applied Biosystems). The obtained sequences were deposited in the

GenBank under accession numbers KF300951 to KF301020 and KX371866 to KX371868.

2.4 | Phylogenetic analysis

Nucleotide sequences of the partial G glycoprotein gene from HRSV-B were edited and aligned with reference strains downloaded from GenBank representing different HRSV-B genotypes using BioEdit v7.0.9.0. The analysis involved 95 nucleotide sequences, with a total of 807 positions in the final database. The most suitable model for nucleotide substitution and associated parameters was inferred using the Modelgenerator V0.85 software. Phylogeny was inferred by maximum likelihood analysis, using the PhyML software. The sequence of the 1967 strain CH18537 was used as outgroup species. Statistical support of the tree nodes was estimated by approximate likelihood ratio test (aLRT). Nucleotide p-distances were calculated in MEGA6.[33]

2.5 | Deduced amino acid

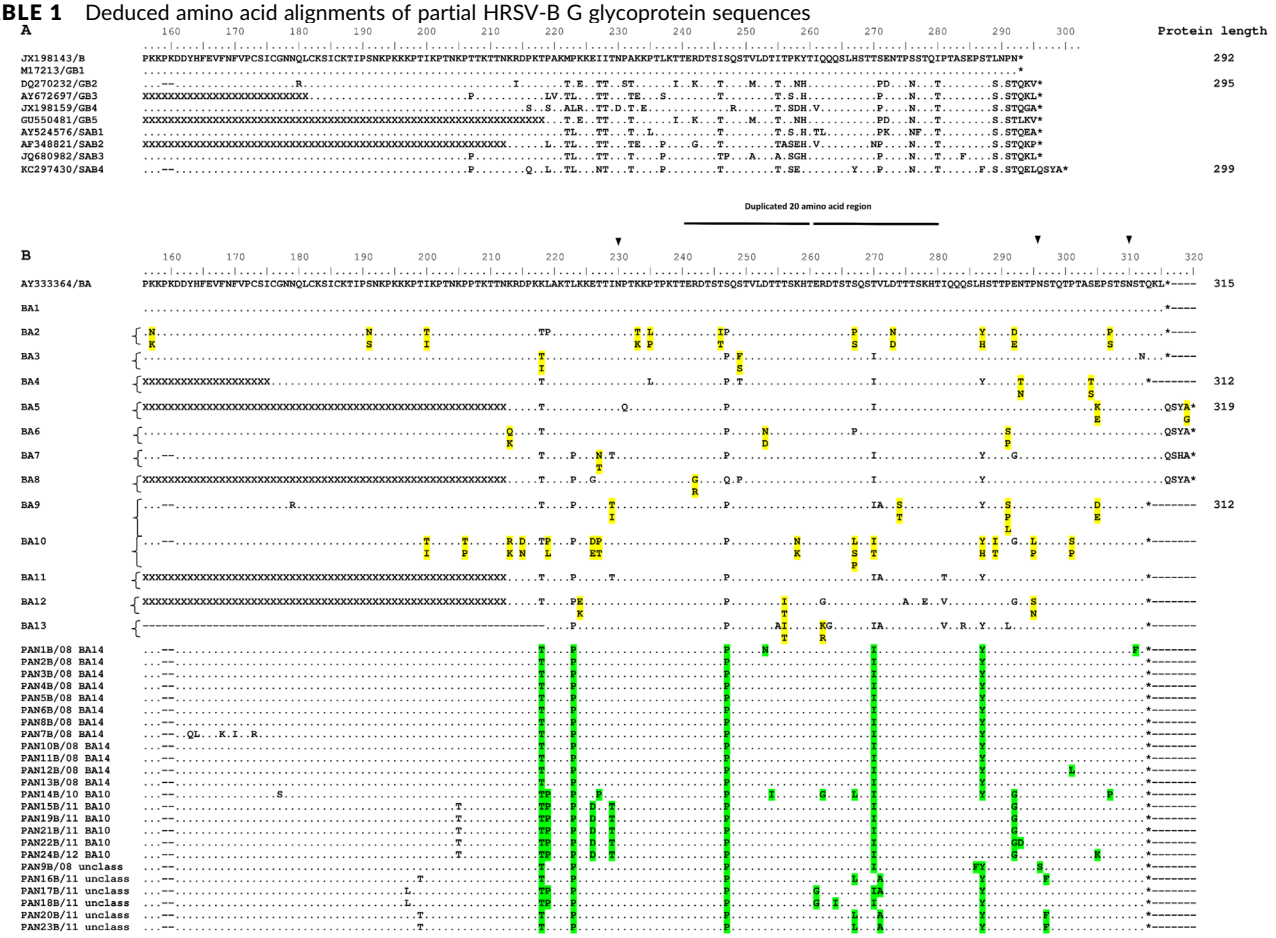
Amino acid sequences derived from the partial G gene nucleotide sequences were deduced by translating nucleotide sequences using the BioEdit v7.0.9.0. software. The amino acid sequences of the ectodomain of the G protein, encompassing amino acid 156 to the C-terminal end of all HRSV-B isolates, were compared with the strain CH18537 (JX198143) which was used as reference for old HRSV-B strain and the strain BA4128/99B (AY333364) as reference for BA RSV-B strains. A consensus aminoacidic sequence was generated for each reference BA genotype, using the strains included in the phylogeny, and intragenotype differences were marked in yellow. For Panamanian strains, aminoacidic changes were marked in green. Differences in deduced protein length and potential N-glycosylation sites were also depicted (Table 1).

3 | RESULTS

3.1 | Clinical and epidemiological information

To assess the genotypes of HRSV circulating in Panama a total of 122 nasopharyngeal swab samples positive to HRSV were randomly selected. One hundred and three samples were amplified, and from them, 75 sequences suitable for analysis were obtained. Sixty sequences corresponded to virus detected in

TABLE 1 Deduced amino acid alignments of partial HRSV-B G glycoprotein sequences



(A) Reference strains without the 20 aminoacid (60-nucleotide) duplication. (B) Reference BA and Panama sequences with the 20 aminoacid duplication. Identical residues are indicated by dots, X mean no sequence data available and asterisks indicates stop codons. Potential N-glycosylation sites are indicated by triangles. Deduced protein lengths are shown on the right. Yellow color indicate diferences in the consensus aminoacid substitutions for the reference BA sequences used in the phylogeny. Green color indicate substitutions in Panama strains refered to AY333364.

hospitalized patients (80%, 60/75) and 15 were from ambulatory patients. All the HRSV positive samples were from children less than or equal to 5 years of age, and 69% (52/75) were younger than 1 year. Of the 60 hospitalized patients, 70% (42/60) were under 1 year of age. Fifty-one sequences (68%) were identified as HRSV-group A and 24 (32%) as HRSV-group B. HRSV-B and HRSV-A co-circulated every year and HRSV-B was predominant in 2008. From the HRSV-B sequences analyzed 13 were from 2008, 1 from 2010, 9 from 2011, and 1 from 2012. Among them, 14 sequences were obtained from hospitalized patients, and 10 from ambulatory patients. Twenty sequences were from infants younger than 12 months of age, three were from infants of 1 year old and one sequence corresponded to a child of 3 years old. Most of the patients arrived with symptoms of an influenza-like illness (ILI) such as cough, fever, and rhinorrhea, however, 14 patients were finally hospitalized, 3 of them with respiratory distress, 1 with broncho-pneumonia, and 3 with bronchiolitis. Bronchiolitis was also diagnosed in three ambulatory patients.

3.2 | Genetic and phylogenetic analysis of HRSV-B

Sequences of HRSV-B were aligned with representative sequences of all previously described group B genotypes. The nucleotide substitution model used for maximum likelihood phylogenetic inference was GTR + I + G and only nodes with support greater than 0.70 were considered significant. Phylogeny of HRSV-B shows that all the Panamanian strains clustered with strains previously assigned to the BA genotype which showed the 60 nucleotide duplication (Fig. 1A,B). Furthermore, Panamanian strains could be further differentiated into different genotypes designated BA10 (6/24, 25%) and a novel genotype, BA14 (12/24, 50%) (Fig. 1B). The criteria to be classified as a separate HRSV genotype is that sequences must cluster together with a support value above 0.70 (or 70%) and with a maximum pairwise distance (*p*-distance) of 0.07 between clustered members.[12,20,22] Strains belonging to the genotype BA14 form a monophyletic group, with high statistical support (aLRT value of 0.92) (Fig. 1B), and the intra group *p*-distance value within the Panamanian BA14 strains was 0.003. Nucleotide distances with other BA genotypes (inter-group *p*-distances) were greater, ranging between 0.014 (BA14 vs BA9) to 0.059 (BA14 vs BA13) (data not shown, available upon request), indicating the presence of a novel genotype. The strains grouped in the new genotype BA14 showed two synonymous nucleotide substitutions at positions 216 (C to T) and 288 (T to C) (data not shown, available upon request).

All strains that clustered in the new genotype BA14 were from 2008 and were the dominant genotype that year. Six strains (five from 2011 and one from 2008 epidemics) remained unclassified. The 2011 strains group together with a hardly significant support (aLRT = 0.73). Nucleotide *p*-distance analysis reveal that these 2011 viruses are more related to BA9 or BA11 strains, meanwhile the PAN9B/2008 is more closely related to the new BA14 genotype (data not shown, available upon request).

3.3 | Amino acid sequence of the G protein from the Panamanian HRSV-B strains

The predicted length of the G protein for all the Panamanian strains from HRSV-B, including the new genotype BA14, was 312 amino acid long (Table 1) due to a premature stop codon.[34] These Panamanian sequences contained the 60-nucleotide duplication described in the BA-like strains and the six-nucleotide deletion at position 490 that resulted in the deletion of proline and lysine (positions 159 and 160, Table 1). This deletion has also been described in some strains from Belgium,[35] Kenya,[36] South Africa,[26] South Korea,[14]India,[37] and China.[16,38] It was also found in BA9,[16,36] BA10,[16] CB-B,[14] and in older genotypes without duplication as SAB4[16] and GB2 (DQ270232 and DQ270233, Genbank direct submission).

Two potential N-glycosylation (Asn-X-Ser/Thr) sites have been described for the ectodomain of the G protein of HRSV BA genotype at amino acid positions 296 and 310,[21,39] with a third putative site at 230.[40] Most Panamanian strains showed the conserved N-glycosylation sites located at amino acid position 296 and 310. As reported, mutations were observed mainly at positions 297 and 311 (Table 1). Only PAN9B/08 has a N296S mutation that changes the Asn-Ser-Thr motif to Ser-Ser-Thr, thus, inhibiting the potential N glycosylation site.

Some amino acid substitutions (K218T, L223P, and S247P) were observed in all the Panamanian strains independently of the specific BA genotype. K218T was also observed in strains from Argentina, Africa (Kenya, South Africa), Asia (Malaysia, China, Japan, Philippines), Pakistan, and Spain (Table 1, Table 2). L223P and S247P were reported similarly in strains from these countries and Senegal (Table 2).

The amino acid substitution at the position T270I was present in almost all Panamanian strains with exception of some unclassified 2011 strains and has been described previously in BA strains from different countries between 2003 to 2015 (Table 2). H287Y substitution was detected in all Panamanian strains except in some strains of BA10 genotype, and was also reported in strains from around the world since 2003 (Table 2). On the other hand, some amino acid changes as L219P, E226D, I229T, E261G, V271A, E292G, S297F, where found only in some Panamanian unclassified strains or some BA10, and were previously reported by other countries (Table 2). The PAN14B/10 strain has four mutations not observed in the other Panamanian strains from the BA10 genotype: T254I, observed in strains from Senegal 2014 and 2015; R262G in Malaysia 2009; T227P and S307P (Table 2). The S267L amino acid change observed in one strain from Spain from 2013 (VH175085/BA10) was present in Panamanian strains of unclassified genotypes with the exception of PAN9B/08, and in PAN14B/10 from BA10 genotype. T264I is present in the strain PAN18B/11 and was previously reported in a 2009 strain belonging to the new genotype BA-C from China. The substitution D253N was found only in the strain PAN1B/08 (BA14) and was observed in one stain from Japan in 2007 (NG-065-07) and one strain from Belgium to 2003 (BE/46/03). E305K was found only in PAN24B/12 (BA10), observed in one strain from Japan in 2005 (NG-095-05). There are some amino acid substitutions detected in only one strain like L286F in

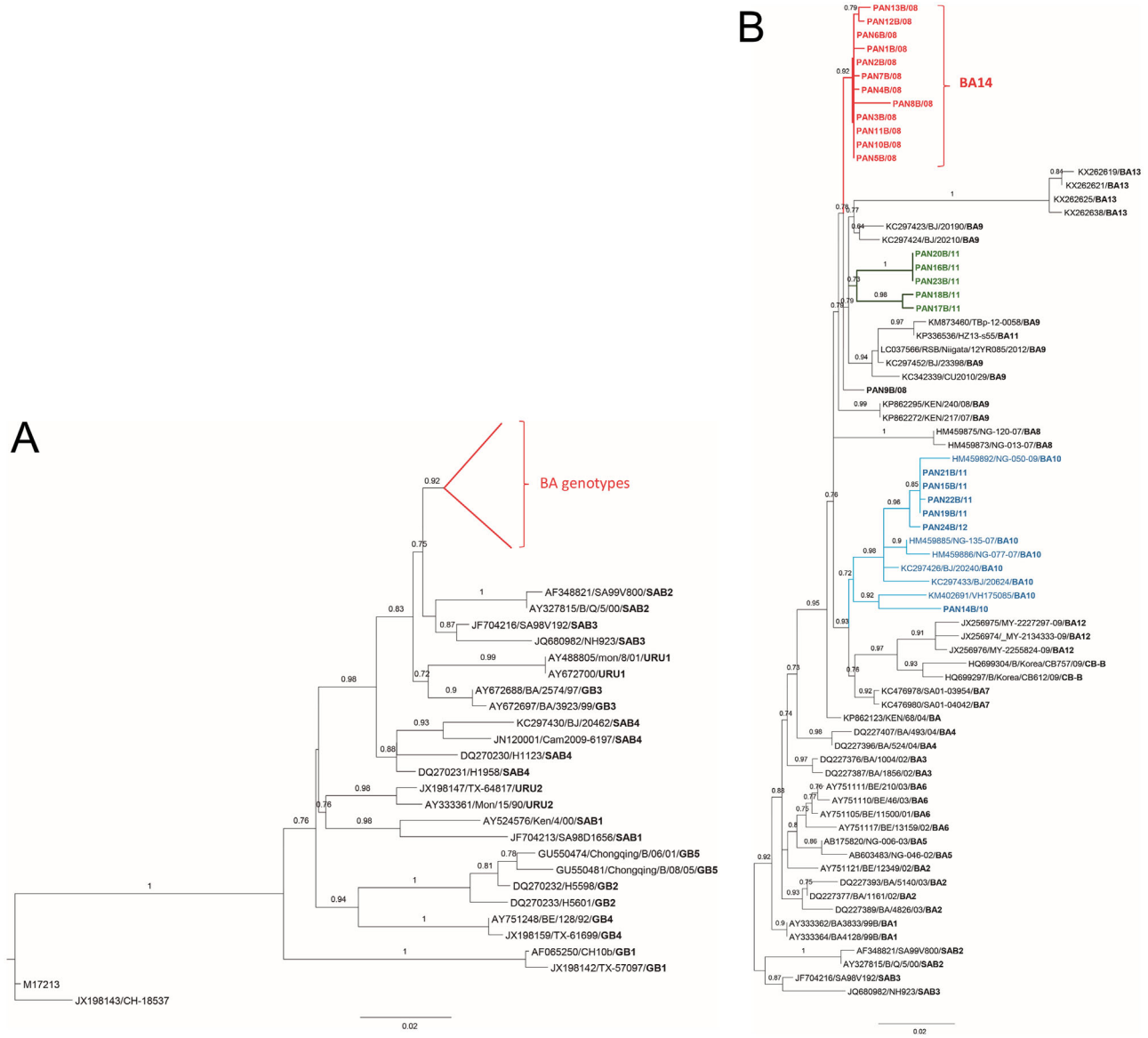


FIGURE 1 ML phylogeny of HRSV-B Panamanian strains and reference sequences from identified genotypes. The tree was constructed using the most suitable model for nucleotide substitution and associated parameters were inferred using the Modelgenerator V0.85 software. Phylogeny was inferred by maximum likelihood analysis, using the PhyML software. The sequence of the 1967 strain CH18537 was used as outgroup species. Statistical support of the tree nodes was estimated by approximate likelihood ratio test (aLRT). Sequences of Panamanian strains (PAN/collection year) were deposited in GenBank under accession numbers KF300951 to KF301020 and KX371866 to KX371868. For visual clarity, the tree was depicted in two panels (A and B). Panel A shows the relationships between old HRSV-B genotypes and the BA genotypes. Nodes corresponding to BA-like genotypes were collapsed. Panel B shows only BA genotypes, Panamanian strains are colored as follows: BA10 in blue, unclassified in green, BA14 in red

PAN9B/08, N293D in PAN22B/11 from the BA10 genotype, and S311F in PAN1B/08 of the BA14 genotype (Table 1, Table 2).

4 | DISCUSSION

HRSV remains as the leading cause of hospitalization due to acute respiratory tract infections, particularly in infants under 5 years of age.[1] Previous reports show that about 90% of infants and young children have experienced HRSV infections by the age of 2 years.[41] Although our sample size is small, of the 24 HRSV-B patients included in the present study, 23 were infants of 1 year old or below, showing that the trend is similar to these previous reports.

Upon its identification in 1999, HRSV-B strains evolved globally to the novel classified BA genotype which became predominant among the recently circulating strains in most countries.[17,23,25,26] A recent study determined that the duplicated region may be involved in the virus attachment to cells. They showed that in vitro, recombinant "BA like" viruses containing the duplicated region in the G protein show an augmented attachment to the cell receptor and have a fitness

TABLE 2 Amino acid substitutions in the G glycoprotein of Panamanian strains in comparison with different HRSV-B genotypes

Amino acid substitution	Panamanian strains	BA genotype of Panamanian strains	Country and year previously reported	BA genotype of previously reported	References
K218T[a]	ALL	BA10, unclassified and BA14	Argentina 2002-2004 Kenya 2008 Malaysia 2009 China 2009 and 2013 South Africa 2010 Japan 2002-2009 Philippines 2012-2013 Pakistan 2012 Spain 2015	All except BA1 strains and strain DQ227387 (BA3)	16,17,19,21,24,25,35,36,49,50
L219P	PAN14B/10, PAN19B/11, PAN15B/11, PAN21B/11, PAN22B/11, PAN24B/12, PAN17B/11 and PAN18B/11	BA10 and unclassified	Argentina 2002-2003 Belgium 2002	BA2, BA10	21,40,44,50
			Japan 2002 and 2009		
			Pakistan, Senegal 2012-2015		
L223P[a]	ALL	BA10, Unclassified and BA14	Spain South Africa Kenya Senegal China Malaysia Japan Philippines 2002-2015	BA7, BA8, BA9, BA10, BA11, BA12, BA13	16,17,21,24,25,36,40
E226D	PAN19B/11, PAN15B/11, PAN21B/11, PAN22B/11 and PAN24B/12	BA10 (except for PAN14B/10)	Japan, China 2009-2011	BA10	16,21
T227P	PAN14B/10	BA10	Spain 2013 (VH175085)	BA10	24
I229T	PAN19B/11, PAN15B/11, PAN21B/11, PAN22B/11 and PAN24B/12	BA10 (except for PAN14B/10)	South Africa 2010 Philippine 2012-2013	BA7, BA9, BA11	17(KC476978);49
S247P[a]	ALL	BA10, unclassified and BA14	Spain South Africa Kenya Senegal China Malaysia Japan Philippines 2002-2015	BA2, BA3, BA4, BA5, BA6, BA7, BA9, BA10, BA11, BA12, BA13	16,17,19,21,24,25,35,36,49
D253N	PAN1B/08	BA14	Japan 2007 Belgium 2003	BA6	21,35
T254I	PAN14B/10	BA10	Senegal 2014-2015	BA9	40
E261G	PAN17B/11, PAN18B/11	Unclassified	Pakistan 2012 India 2009-2012	BA9	37,50
R262G	PAN14B/10	BA10	Malaysia 2009	BA12	25
T264I	PAN18B/11	BA11	China 2009	BA-C	16
S267L	PAN16B/11, PAN20B/11, PAN23B/11 and PAN14B/10	BA10 and unclassified	Spain 2013 (VH175085)	BA10	24
T270I[a]	ALL (except for PAN16B/11, PAN20B/11 and PAN23B/11)	BA10, unclassified and BA14	Argentina, South Africa, Kenya, China, Japana, Philippines, Senegal, Pakistan 2003-2015	BA3, BA4, BA5, BA7, BA8, BA9, BA10, BA11, BA13	16,19,21,24,40,49,50
V271A	PAN16B/11, PAN17B/11,	Unclassified	Pakistan 2012	BA9, BA11, BA13	16,40,50,51

(Continues)

TABLE 2 (Continued)

Amino acid substitution	Panamanian strains	BA genotype of Panamanian strains	Country and year previously reported	BA genotype of previously reported	References
	PAN20B/11 and PAN23B/11		Kenya 2008 China 2010 Senegal 2012-2015		
L286F	PAN9B/08	Unclassified			Only in Panamanian strains
H287Y[a]	ALL (except for BA10)	Unclassified, PAN14B/10 and BA14	Argentina 2004 Japan 2003-2009 Kenya 2008 South Africa 2010 Philippines, Senegal, Pakistan 2012-2015	BA2, BA4, BA7, BA8, BA9, BA10, BA11, BA13	17,19,21,36,40,49,50
E292G	PAN14B/10, PAN19B/11, PAN15B/11, PAN21B/11, PAN22B/11 and PAN24B/12	BA10	Japan 2007 Malaysia 2009 India 2009-2010	BA7, BA10, BA12	44
N293D	PAN22B/11	BA10			Only in Panamanian strains
N296S	PAN9B/08	Unclassified	USA	CH18537 (not BA genotype)	52
S297F	PAN16B/11, PAN20B/11 and PAN23B/11	Unclassified	Japan 2009	BA9	21
P301L	PAN12B/08	BA14	USA	NY97 (not BA genotype)	11
E305 K	PAN24B/12	BA10	Japan 2005	BA5	21
S307P	PAN14B/10	BA10	Argentina 2003 (BA/5140/03) India 2010	BA2 BA9	19,37
S311F	PAN1B/08	BA14			Only in Panamanian strains

[a]Common, amino acid substitution.

advantage compared to viruses without the duplication.[42] All analyzed HRSV-B strains from Panama clustered with strains of the BA genotype which have the 60-nt duplication in the second hypervariable region of the G gene, confirming that this is currently the predominant genotype worldwide for HRSV-B.

The BA virus evolved rapidly because of the global spread to at least 13 new BA genotypes (BA1-BA13). The 18 Panamanian BA strains could be further classified in the BA10 genotype, and the here reported BA14 genotype. Another six strains could not be genotyped. From them, PAN9B/08 resulted more similar to BA14 strains with a *p*-distance value of 0.007. Another six strains (PAN16B/11, PAN17B/11, PAN18B/11, PAN20B/11, and PAN23B/11) group together, but with a barely significant support (0.73). When analyzing the *p*-distances they exhibit a mean intra-group distance of 0.023, but three of them (PAN16B/11, PAN20B/11, and PAN23B/11) are more closely related to BA9, while another two (PAN17B/11, PAN18B/11) are closer to BA11 strains. The addition of new HVRS B sequences may clarify the relationships between these Panamanian strains. It was yet described that groups with poor support can consolidate into new genotypes by the addition of new sequences from different locations or more recent epidemics, or, on the contrary, these unclassified viruses may split into pre-existing genotypes.[11,43] The new genotype BA14 is a monophyletic group with a highly significant statistical support (aLRT = 0.92) and is genetically homogeneous, showing an intra group *p*-distance of 0.003. This fulfills the criteria to assign a new genotype in HRSV. Regarding the other BA genotypes, BA9 is the most related to BA14, with a *p*-distance of 0.014 and BA13 is the more distant, with a *p*-distance of 0.059. The BA14 genotype was strongly predominant in 2008, but apparently extinguished in the next epidemic seasons. Of the 12 samples that were grouped in the new genotype BA14, 10 were from the province of Panama and two from the central provinces (PAN12B/08, Cocle and PAN13B/08, Los Santos) and were collected during the months of July to November 2008. BA10 and unclassified samples were also mainly from the province of Panama and from the same months. Although most of them came from the province of Panama, where 44% of the population is located, they came from different regions and hospitals. Thus, there is probably no epidemiological relation between BA14, BA10, and unclassified confirmed cases.

BA9 and BA10 were first identified during the years 2006-2008 in Japan and became predominant BA genotypes during the time frame

2008-2010 at least in Asia.[14,21,44] This corresponds to the time frame of their circulation in Panama; however, BA9 did not appear to be predominant in Panama during the years analyzed, with the exception of some unclassified "BA9 like" strains. On the other hand, the genotype BA10 was observed from 2010 to 2012, just after its expansion worldwide. Our findings on the circulation of multiple genotypes in the same year are consistent with the circulation pattern reported for HRSV in different studies. Also, multiple HRSV genotypes from group A and B co-circulated in a single epidemic season, and different genotypes can dominate in consecutive seasons with one or two dominant genotypes being replaced in successive years.[10,11,43,45,46] Future studies including samples from previous years are needed to determine the precise time of introduction of the different genotypes in Panama, especially the new BA14 genotype, as well as analyze the phylogeography of HRSV in Panama and the Americas.

Differences in the length of the deduced G protein sequences among the HRSV-B strains result of the comparison with the reference old and BA strains (292, 293, 295, 296, 297, 299, 312, 315, and 319 amino acids) that are likely caused by the usage of alternative termination codons through the presence of in-frame duplications, deletions, and insertions. One frameshift mutation and premature stop codon are thought to be associated with antigenic variation in HRSV escape mutants that recognize strain-specific epitopes.[7] These changes were observed in the Panamanian BA strains and might enhance the fitness of the viruses, which could explain why BA genotypes are becoming dominant worldwide and replacing all other HRSV-B genotypes. This probably contributes to the evasion of the immune response of the population and may even complicate vaccine development.[17,26]

Twenty-five amino acid substitutions were observed in group B Panamanian strains; from them, 22 have been previously reported in different studies and only 3 were exclusively observed in Panamanian strains (Table 2). Several of these amino acid sites (219, 227, 247, 267, 270, 286, 287, 292, 297, 305, and 311) have been found to be under positive selection for at least two methods.[34,47,48] Different authors hypothesize that this amino acid changes could be involved in the evasion of the immune system response; further research is needed to explore possible evolutionary advantages that these changes may confer to these viruses.

Limitations in this study are the reduced number of analyzed samples, and that the samples were not representative of all months during the studied years. Despite these limitations, this is the first molecular analysis on HRSV-B in Central America which reports the detection in Panama of strains belonging to different BA genotypes, one of them being the novel HRSV group B genotype BA14. Our analysis also provides information regarding the prevalent genotypes of HRSV in Panama in the 2008-2012 period.

ACKNOWLEDGMENTS

We thank to Melissa Gaitán and José Valenzuela for helping with processing and the samples extraction in the Department of Research in Virology and Biotechnology, Gorgas Memorial Institute of Health Studies. Also, Néstor Sosa and Alexander Martínez from Gorgas Memorial Institute of Health Studies for all your help in this study. Secretaría Nacional de Ciencias, Tecnología e Innovación Panamá (SENACYT); Grant number: CAP10-001 (JA). Agencia Nacional de Investigación e Innovación y Programa de Desarrollo de las Ciencias Básicas del Uruguay. Dr. Juan Miguel Pascale and Dr. Sandra López Vergès are distinguished members of the Sistema Nacional de Investigación (SNI) that is supported by the Secretaría Nacional de Ciencias, Tecnología e Innovación Panamá (SENACYT).

REFERENCES

1. Nair H, Nokes DJ, Gessner BD, et al. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. *Lancet*. 2010;375:1545–1555.
2. Glezen WP, Taber LH, Frank AL, Kasel JA. Risk of primary infection and reinfection with respiratory syncytial virus. *Am J Dis Child*. 1986;140:543–546.
3. Weber MW, Mulholland EK, Greenwood BM. Respiratory syncytial virus infection in tropical and developing countries. *Trop Med Int Health*. 1998;3:268–280.
4. Collins PL, McIntosh K, Chanock RM. Respiratory syncytial virus. In: Fields BN, Knipe DM, Howley PM, eds. *Fiels Virology*. Philadelphia: Lippincott-Raven, Inc.; 1996:1313–1514.
5. Levine S, Klaiber-Franco R, Paradiso PR. Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. *J Gen Virol*. 1987;68:2521–2524.
6. Walsh EE, Brandriss MW, Schlesinger JJ. Purification and characterization of the respiratory syncytial virus fusion protein. *J Gen Virol*. 1985;66:409–415.
7. Melero JA, Garcia-Barreno B, Martinez I, Pringle CR, Cane PA. Antigenic structure, evolution and immunobiology of human respiratory syncytial virus attachment (G) protein. *J Gen Virol*. 1997;78:2411–2418.
8. Peret TC, Hall CB, Schnabel KC, Golub JA, Anderson LJ. Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. *J Gen Virol*. 1998;79:2221–2229.
9. Anderson LJ, Hierholzer JC, Tsou C, et al. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. *J Infect Dis*. 1985;151:626–633.
10. Galiano MC, Palomo C, Videla CM, Arbiza J, Melero A, Carballal G. Genetic and antigenic variability of human respiratory syncytial virus (groups a and b) isolated over seven consecutive seasons in argentina (1995 to 2001). *J Clin Microbiol*. 2005;43:2266–2273.
11. Peret TC, Hall CB, Hammond GW, et al. Circulation patterns of group A and B human respiratory syncytial virus genotypes in 5 communities in North America. *J Infect Dis*. 2000;181:1891–1896.
12. Venter M, Madhi SA, Tiemessen CT, Schoub BD. Genetic diversity and molecular epidemiology of respiratory syncytial virus over four consecutive seasons in South Africa: identification of new subgroup A and B genotypes. *J Gen Virol*. 2001;82:2117–2124.
13. Shobugawa Y, Saito R, Sano Y, et al. Emerging genotypes of human respiratory syncytial virus subgroup a among patients in Japan. *J Clin Microbiol*. 2009;47:2475–2482.
14. Baek YH, Choi EH, Song M-S, et al. Prevalence and genetic characterization of respiratory syncytial virus (RSV) in hospitalized children in Korea. *Arch Virol*. 2012;157:1039–1050.
15. Eshaghi A, Duvvuri VR, Lai R, et al. Genetic variability of human respiratory syncytial virus A strains circulating in Ontario: a novel genotype with a 2 nucleotide G gene duplication. *PLoS ONE*. 2012;7:e32807.
16. Cui G, Zhu R, Qian Y, et al. Genetic variation in attachment glycoprotein genes of human respiratory syncytial virus subgroups a and b in children in recent five consecutive years. *PLoS ONE*. 2013;8:1–13.

17. Pretorius MA, Van Niekerk S, Tempia S, et al. Replacement and positive evolution of subtype a and b respiratory syncytial virus g-protein genotypes from 1997-2012 in South Africa. *J Infect Dis*. 2013;208:S227–S237.
18. Trento A, Ábrego L, Rodriguez-Fernandez R, et al. Conservation of G protein epitopes in respiratory syncytial virus (Group a) despite broad genetic diversity: is antibody selection involved in virus evolution? *J Virol*. 2015;89:7776–7785.
19. Trento A, Viegas M, Galiano M, et al. Natural history of human respiratory syncytial virus inferred from phylogenetic analysis of the attachment (g) glycoprotein with a 60-nucleotide duplication natural history of human respiratory syncytial virus inferred from phylogenetic analysis of the Attachment (G) Glycoprotein with a 60-Nucleotide Duplication. *J Virol*. 2006;80:975–984.
20. Blanc A, Delfraro A, Frabasile S, Arbiza J. Genotypes of respiratory syncytial virus group B identified in Uruguay. *Arch Virol*. 2005;150:603–609.
21. Dapat IC, Shobugawa Y, Sano Y, et al. New genotypes within respiratory syncytial virus group B genotype BA in Niigata, Japan. *J Clin Microbiol*. 2010;48:3423–3427.
22. Arnott A, Vong S, Mardy S, et al. A study of the genetic variability of human respiratory syncytial virus (HRSV) in Cambodia reveals the existence of a new HRSV group B genotype. *J Clin Microbiol*. 2011;49:3504–3513.
23. Trento A, Casas I, Calderón A, et al. Ten years of global evolution of the human respiratory syncytial virus BA genotype with a 60-nucleotide duplication in the G protein gene. *J Virol*. 2010;84:7500–7512.
24. Gimferrer L, Andrés C, Campins M. Circulation of a novel human respiratory syncytial virus group B genotype during the 2014-2015 season in Catalonia (Spain). *Clin Microbiol Infect*. 2015;448:11–14.
25. Khor CS, Sam IC, Hooi PS, Chan YF. Displacement of predominant respiratory syncytial virus genotypes in Malaysia between 1989 and 2011. *Infect Genet Evol*. 2013;14:357–360.
26. van Niekerk S, Venter M. Replacement of previously circulating respiratory syncytial virus subtype B Strains with the BA genotype in South Africa. *J Virol*. 2011;85:8789–8797.
27. Laguna-Torres VA, Gamero E, Halsey ES, Tadeusz J. Influenza and other respiratory viruses in three Central American countries. *Influenza Other Respir Viruses*. 2010;5:123–134.
28. Mccracken JP, Prill MM, Arvelo W, et al. Respiratory syncytial virus infection in guatemala 2007-2012. *J Infect Dis*. 2013;208:S197–S206.
29. Schlaudecker EP, Heck JP, Macintyre ET, et al. Etiology and seasonality of viral respiratory infections in rural Honduran children. *NIH Public Access*. 2013;31:1113–1118.
30. Sovero M, Garcia J, Kochel T, et al. Circulating strains of human respiratory syncytial virus in Central and South America. *PLoS ONE*. 2011;6:e22111.
31. Valdés O, Ramírez V, Savón LC, Goyenechea Á, Valdivia Á, González LG. Diferentes patrones de circulación dentro de los subgrupos A y B del virus sincitial respiratorio humano en algunas provincias de Cuba. *Rev Cubana Med Trop*. 2004;56:94–102.
32. Parveen S, Sullender WM, Fowler K, Lefkowitz EJ, Kapoor SK, Broor S. Genetic variability in the G protein gene of group A and B respiratory syncytial viruses from India. *J Clin Microbiol*. 2006;44:3055–3064.
33. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol*. 2013;30:2725–2729.
34. Botosso VF, Zanotto PMDA, Ueda M, et al. Positive selection results in frequent reversible amino acid replacements in the G protein gene of human respiratory syncytial virus. *PLoS Pathog*. 2009;5:e1000254.
35. Zlateva KT, Lemey P, Moës E, Van Ranst M, Moe E. Genetic variability and molecular evolution of the human respiratory syncytial virus subgroup B attachment G protein genetic variability and molecular evolution of the human respiratory syncytial virus subgroup b attachment g protein. *J Virol*. 2005;79:9157.
36. Agoti CN, Otieno JR, Ngama M, et al. Successive respiratory syncytial virus epidemics in local populations arise from multiple variant introductions, providing insights into virus persistence. *J Virol*. 2015;89:11630–11642.
37. Choudhary ML, Anand SP, Wadhwa BS, Chadha MS. Genetic variability of human respiratory syncytial virus in Pune, Western India. *Infect Genet Evol*. 2013;20:369–377.
38. Ren L, Xiao Q, Zhou L, Xia Q, Liu E. Molecular characterization of human respiratory syncytial virus subtype B: a novel genotype of subtype B circulating in China. *J Med Virol*. 2015;87:1–9.
39. Agrawal AS, Sarkar M, Ghosh S, et al. Prevalence of respiratory syncytial virus group B genotype BA-IV strains among children with acute respiratory tract infection in Kolkata, Eastern India. *J Clin Virol*. 2009;45:358–361.
40. Fall A, Dia N, Hadj E, Kader A, Kiori DE. Epidemiology and molecular characterization of human respiratory syncytial virus in senegal after four consecutive years of surveillance 2012-2015. *PLoS ONE*. 2016;11:e0157163.
41. Simoes EA. Respiratory syncytial virus infection. *Lancet (London, England)*. 1999;354:847–852.
42. Hotard AL, Laikhter E, Brooks K, Hartert TV, Moore ML. Functional analysis of the 60-nucleotide duplication in the respiratory syncytial virus Buenos Aires strain attachment glycoprotein. *J Virol*. 2015;89:8258–8266.
43. Frabasile S, Delfraro A, Facal L, et al. Antigenic and genetic variability of human respiratory syncytial viruses (group A) isolated in Uruguay and Argentina: 1993-2001. *J Med Virol*. 2003;71:305–312.
44. Ohno A, Suzuki A, Lupisan S, et al. Genetic characterization of human respiratory syncytial virus detected in hospitalized children in the Philippines from 2008 to 2012. *J Clin Virol*. 2013;57:59–65.
45. Arbiza J, Delfraro A, Frabasile S. Molecular epidemiology of human respiratory syncytial virus in Uruguay: 1985–2001. *Mem Inst Oswaldo Cruz*. 2005;100:221–230.
46. Viegas M. Molecular epidemiology of respiratory syncytial virus in pediatric patients in a six-year period. *Acta Bioquím Clín Latinoam*. 2011;45:3–45.
47. Nagasawa K, Hirano E, Kobayashi M, et al. Molecular evolution of the hypervariable region of the attachment glycoprotein gene in human respiratory syncytial virus subgroup B genotypes BA9 and BA10. *Infect Genet Evol*. 2015;36:217–223.
48. Esposito S, Piralla A, Zampiero A, et al. Characteristics and their clinical relevance of respiratory syncytial virus types and genotypes circulating in northern italy in five consecutive winter seasons. *PLoS ONE*. 2015;10:e0129369.
49. Malasao R, Okamoto M, Chaimongkol N, et al. Molecular characterization of human respiratory syncytial virus in the Philippines 2012-2013. *PLoS ONE*. 2015;10:e0142192.
50. Aamir UB, Alam MM, Sadia H, Sohail S, Zaidi Z. Molecular characterization of circulating respiratory syncytial virus (RSV) genotypes in gilgit baltistan province of pakistan during 2011-2012 winter season. *PLoS ONE*. 2013;8:e74018.
51. Agoti CN, Mbisa JL, Bett A, et al. Intrapatient variation of the respiratory syncytial virus attachment protein gene. *J Virol*. 2010;84:10425–10428.
52. Tapia LI, Shaw CA, Aideyan LO, et al. Gene sequence variability of the three surface proteins of human respiratory syncytial virus (HRSV) in Texas. *PLoS ONE*. 2014;9:e90786.

How to cite this article: Ábrego LE, Delfraro A, Franco D, et al. Genetic variability of human respiratory syncytial virus group B in Panama reveals a novel genotype BA14. *J Med Virol*. 2017;9999:1–9. https://doi.org/10.1002/jmv.24838

CAPÍTULO III

CAPÍTULO III

CARACTERIZACIÓN MOLECULAR DE CEPAS DE METAPNEUMOVIRUS HUMANO AISLADOS EN PANAMÁ DURANTE LOS AÑOS 2008-2012.

MANUSCRITO: GENOTYPES OF HUMAN METAPNEUMOVIRUS CIRCULATING DURING 2010-2012 IN CHILDREN FROM PANAMA

Leyda E. Ábrego[1], Santiago Mirazo[4], Adriana Delfraro[4], Danilo Franco[1,3], Marlene Castillo[1], Melissa Gaitán[1], Juan Castillo[2], Brechla Moreno[1], Juan M. Pascale[2,3] and Juan Arbiza[4]

Este estudio reporta los genotipos de Metapneumovirus humano (HMPV) que circularon en Panamá en niños ≤ 5 años durante el 2010-2012. Se utilizó el gen N, que a pesar de ser altamente conservado ha demostrado la circulación de todos los linajes y sub-linajes de HMPV y en este estudio no fue la excepción. Se mostró la circulación de 4 sub-linajes. Los datos epidemiológicos basados en los tres años de estudio no mostraron un patrón estacional dela circulación para este virus en Panamá.

---

[1]Deparment of Research in Virology and Biotechnology, Gorgas Memorial Institute of Health Studies, Panama City, Panama; [2]Department of Genomic and Proteomic, Gorgas Memorial Institute of Health Studies, Panama City, Panama; [3]School of Medicine, University of Panama, Panama City, Panama; [4]Virology Section, School of Sciences, University of the Republic, Montevideo, Uruguay.

Correspondence to: Juan Arbiza, Sección de Virología, Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo 11400, Uruguay. E-mail: jarbiza@fcien.edu.uy

Short Communication

Genotypes of Human Metapneumovirus circulating during 2010-2012 in children from Panama†

Shortened Title: Human Metapneumovirus in Panama

Leyda E. Ábrego[1], Santiago Mirazo[4], Adriana Delfraro[4], Danilo Franco[1,3], Marlene Castillo[1], Melissa Gaitán[1], Juan Castillo[2], Brechla Moreno[1], Juan M. Pascale[2,3] and Juan Arbiza[4]

[1]Deparment of Research in Virology and Biotechnology, Gorgas Memorial Institute of Health Studies, Panama City, Panama;

[2]Department of Genomic and Proteomic, Gorgas Memorial Institute of Health Studies, Panama City, Panama;

[3]School of Medicine, University of Panama, Panama City, Panama;

[4]Virology Section, School of Sciences, University of the Republic, Montevideo, Uruguay.

Correspondence to: Juan Arbiza, Sección de Virología, Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo 11400, Uruguay. E-mail: jarbiza@fcien.edu.uy

†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/jmv.24969]

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

Received 17 July 2017; Revised 25 August 2017; Accepted 21 September 2017
Journal of Medical Virology

DOI 10.1002/jmv.24969

Abstract

Human metapneumovirus (HMPV) is a common causative agent of severe respiratory tract infections in children under 5 years old, the elderly and immunocompromised patients, being responsible for 5-15% of all viral respiratory infections requiring hospitalization. Though HMPV was included in the surveillance program for respiratory viruses in 2010, its genotype distribution remains unknown. Herein, 45 positive samples to HMPV from children ≤5 years old were characterized by phylogenetic analysis based on N gene sequence. Results showed the co-circulation of four sub-lineages: A2a (8.8%), A2b (55.5%), B1 (15.6%) and B2 (20%), demonstrating the genetic heterogeneity of HMPV circulating in Panamá.

Key Words: human metapneumovirus, nucleoprotein, genotype, acute respiratory infections (ARI).

Introduction

Human metapneumovirus (HMPV) was first observed in isolates of nasopharyngeal aspirate samples collected from children with lower respiratory tract infection during the 90's in Netherlands.[1] Serological surveys revealed a 100% seroprevalence in samples of patients between an age ranging 8-99 years since 1958, indicating that the virus has been circulating in the human population for more than 43 years. Combined virological data indicated that the newly virus was member of the *Paramyxoviridae* family and phylogenetic analyses showed that it was related to *avian pneumovirus* C (APV-C).[1,2] Further studies showed that HMPV affects all age groups who generally presents mild clinical manifestations, including cough, dyspnea, wheezing/stridor, concomitant otitis media; being more severe producing bronchiolitis and pneumonia, accompanied by fever, myalgia and vomiting in very young children, the elderly, and immunocompromised patients who can develop fatal complications.[3,4,5] Last ICTV report classifies this group of viruses in the *Metapneumovirus* genus of the *Pneumoviridae* family. [6]

Two major genetic lineages, A and B have been described for HMPV, based in differences in virus neutralization titers and phylogenetic analyses of complete N, P, M, F, G and L genes. [1,7] Each lineage could be further divided into two genetic subgroups (A1 and A2 in the lineage A; and B1 and B2 in the lineage B).[8] Later, the A2 subgroup has been divided in two clades (A2a and A2b).[9] After its discovery in Netherlands, HMPV, has been reported in other European countries,[10] Asia[11,12] and North America.[7,13] HMPV has been also reported in South American countries, as Brazil,[14] Argentina,[15] Uruguay [16,17] and Chile. [18] In Central America by contrast, very little is known regarding HMPV epidemiology[19] and only Costa Rica,[20] Colombia,[21] Nicaragua, Honduras and Guatemala have reported the detection and/or the molecular characterization of HMPV. [22,23]

Gorgas Memorial Institute of Health Studies (GMI) from Panama has been certified since 2007 by the World Health Organization (WHO) as the National Influenza Center (NIC). Since 1976, the GMI has a surveillance program for Influenza and other respiratory viruses which mainly receives samples of nasopharyngeal swabs coming from patients with influenza-like illness (ILI) and severe acute respiratory infections (SARI) from 18 sentinel sites located throughout the country. At the end of 2010, the surveillance of HMPV was included as part of this program and according to data recorded by this Center, by 2011 HMPV showed a frequency of 1.3% in children ≤ 5 years of age, which increased in 2012 to 13.9%. The seasonality of HMPV has been previously reported with a major peak during the winter months, concurrent or after the respiratory syncytial virus (RSV) epidemic season[3]; showed seasonal peaks in spring were also reported.[24] In Central America seasonality is not clearly defined. Even though several studies have reported seasonality patterns mainly during the rainy season[21], minor peaks in different months during the year seem to occur.[19,23] To date, nevertheless, there has been no description of circulating HMPV genotypes in the country. Different HMPV genome regions have been used for viral genotyping, but being N, G and F genes are mostly used, with comparable clustering outcomes.[9,11,1214,17] Incongruences in lineage assignments have been reported when comparing genes F and G.[25] Phylogenetic analyses of the N and F genes have detected the four lineages and sublineages of HMPV showing the same tree topology.[9,11,25] Although the N gene is highly conserved, the novel sublineage A2a was described by the analysis of this gene and has been used in several reports.[9,14,17] Herein we reported the genetic characterization of HMPV based in the N gene of strains obtained from children ≤ 5 years of age.

Methods

Two hundred and sixteen nasopharyngeal swabs positive to HMPV from children ≤5 years of age, collected from end August 2010 to December 2012 through the surveillance program for Influenza and other respiratory viruses were included in this study. All positive samples from end 2010 (29), and the complete year 2011 (11) were analyzed. Regarding 2012, one hundred and ninety two samples resulted positive to HMPV, but only 176 were available to our laboratory. Protocol was submitted and approved by the Gorgas Memorial Institute Bioethics Committee (no. 084/CBI/ICGES/12 and no. 617/CBI/ICGES/13). Viral RNA was extracted from 140 µl of nasopharyngeal swabs using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. In order to detect the viral genome, a retrotranscription followed by PCR (RT-PCR) was performed to amplify the N gene of HMPV, as described by Bastien.[13] The amplified products were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Sequencing of both strands was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the same primers used in the RT-PCR. BigDye® XTerminator™ Purification Kit was used for purification of the sequence reactions and then sequenced in a 3130xl genetic analyzer (Applied Biosystems). The obtained sequences were deposited in the GenBank under the accession numbers MF325370 to MF325414.

Nucleotide sequences of the partial N nucleoprotein gene from HMPV were edited and aligned with reference strains representative of different genetic linages using BioEdit v7.0.9.0. software. The analysis involved nucleotide (1094 nucleotides-length) sequences, corresponding to the Panamanian strains and 9 were representative reference sequences of the genotypes (see supplementary material). Phylogenetic relationships were inferred by

using the Maximum Likelihood criterion, and the General Time Reversible model of nucleotide substitution. Statistical support of the nodes was calculated through 2000 bootstrap replicates; only nodes with support above 90 % were considered significant; MEGA6 software was used for tree inference and support calculations.[26, 27]

Results and Discussion

Forty-five sequences of N gene from HMPV Panamanian strains were analyzed. Of these, 10 were from samples collected in 2010, 6 in 2011 and 29 in 2012. Since HMPV was included in the surveillance program of respiratory virus at the end of August 2010, 29 samples were detected in the first year of the study. However, despite that by 2011, surveillance for HMPV was performed throughout the year, only 11 samples were positive for this virus from the total samples received. This observation suggests an apparent low frequency or circulation. By contrast, by the year 2012, HMPV had a remarkable increase, since 229 samples (including adult and infant patients) were positive to this virus, placing it as the third most frequent virus in that year. Regarding 2012 epidemics, it must be pointed out that of the 229 HMPV positive cases, 192 were from children ≤ 5 years of age. From them, 176 samples were available for this study and N gene could be amplified only in 88 of these samples. Additionally, several 2012 N sequences were identical, and some had low quality. Because of that, and to avoid sampling bias, 29 sequences from 2012 epidemics were finally included in the dataset for phylogeny. Overall, these results agree with reports on high and low frequencies of HMPV in alternating years (Falsey[5] and Huck[9]). Epidemiological surveillance for HMPV in Panama, performed during 2010, began at the end of August. Most of the positive cases for this year were concentrated in the months of September, October and November. In December 2010, only 3 cases were detected.

Although by 2011 this virus was surveilled throughout the year, few cases were detected, and most were during the month of December. In 2012 the circulation of hMPV was high. Cases were detected during all months of this year, but the highest frequency was during the period May-August. Because surveillance in 2010 was not complete, we cannot discuss HMPV circulation during this year. But, based on the results of the complete epidemiological surveillance during 2011 and 2012, we are not able to infer a seasonal trend of HMPV in our country. These results are consistent with those obtained in Guatemala in a 5-year study (November 2007-December 2012) where a clear seasonality for HMPV infection could not be evidenced.[23] By contrast, results obtained by Schlaudecker et al. (2012) in Honduras,[22] a clear peak was reported in the period July - October and during the raining season in Colombia.[21] In this work, all selected samples were from children ≤ 5 years of age, of which 80% (36/45) were infants ≤ 1 year old. Among them, 37 sequences were obtained from hospitalized patients, 6 from ambulatory patients and 2 from samples which this data was not recorded. Common clinical picture of the patients was an influenza-like illness (ILI) with cough, fever, and rhinorrhea as main symptoms. Six patients ≤ 3 years had pneumonia, 6 had bronchiolitis and two exhibited bronchopneumonia. Our data on the highest number of positive cases in children ≤1 are consistent with those reported by Williams,[28] who found the highest number of children infected with HMPV among infants under six months and up to one year of age. As for the most frequently encountered symptoms, fever and cough are consistent with those of higher frequency reported by Boivin et al., in 2002 in Canada[3] and García et al., in a study from Latin America.[19] Phylogenetic analysis performed with the N gene identified the co-circulation of four HMPV sub-lineages, A2a (4/45, 8.8%), A2b (25/45, 55.5%), B1 (7/45, 15.6%) and B2 (9/45, 20%). Interestingly, the sub-lineage A1 was not represented in this

study (Fig. 1). Data presented here is consistent with the history of HMPV discovery. After Van den Hoogen characterization and description of the sub-lineages A1, A2, B1 and B2,[8] they have been later extensively reported in different studies from Germany,[9] India,[25] China,[12] Italy,[29] among other countries. By contrast, strains from the sub-lineage A1 have been described in very few reports, being identified for the last time in 2008.[10] On the other hand, the sub-lineages A2a and A2b, initially classified as strains in 2003-2004 in Germany,[9] were reported to circulate more frequently, with a clear predominance of A2b sub-lineage.[12,30] These results completely agree with those obtained in our study, since we found a wide circulation of subgroup A2, B1 and B2, but not of A1 strains. In addition, the A2b sub-lineage was the most frequently found in this work, as reported elsewhere.[30] Most of the Panamanian strains from sub-lineages A2a and A2b were samples collected in the 2012 epidemic. Strains from the sub- lineage B1, in turn, were all obtained in 2010, while samples belonging to sub- linage B2 were collected during all the period of the study (2010-2012). This interesting temporal pattern observed needs to be further investigated and its epidemiological implications remains unknown.

The main limitation of this study was the number of samples we managed to sequence and the incomplete surveillance of this virus during 2010. Additional information from a more extended period is needed to assess the seasonality pattern of this virus in Panama, as well as it is important to analyze G gene to add information on the genetic variability of circulating strains. However, this report is interesting since it represents the second study reporting HMPV genotypes in Central America. To conclude, data regarding HMPV genotypes and sub-lineages distribution in Central America is still scarce, even more, considering the increasing incidence of this viral infection in ARI in children and immunocompromised. Herein, the co-circulation in the population of multiple sub-lineages

is reported, thus suggesting that the molecular epidemiology of HMPV is more complex than the initially thought.

Acknowledgments

Financial support:

Secretaría Nacional de Ciencias, Tecnología e Innovación Panamá (SENACYT); Grant number: CAP10-001 (JA). Agencia Nacional de Investigación e Innovación y Programa de Desarrollo de las Ciencias Básicas del Uruguay. Dr. Juan Miguel Pascale distinguished members of the National Research System that is supported by the Secretaría Nacional de Ciencias, Tecnología e Innovación Panamá (SENACYT).

References:

1. van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, et al A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med. 2001;7:719–724.
2. van den Hoogen BG, Bestebroer TM, Osterhaus AD, Fouchier1 RA. Analysis of the Genomic sequence of a human metapneumovirus. Virology. 2002;295:119–132
3. Boivin G, Abed Y, Pelletier G, Ruel L, Moisan D, Cote S, Peret TC, Erdman DD, Anderson LJ. Virological features and clinical manifestations associated with human metapneumovirus: a new paramyxovirus responsible for acute respiratory-trac infections in all age groups. J Infect Dis. 2002; 186:1330–1334.
4. Sharmeen Samuel, MD, Sowmya Nanjappa, MBBS, MD, Christopher D. Cooper, MD, and John N. Greene, MD. Human metapneumovirus infection in immunocompromised patients. Cancer Control. 2016; 23:442-445.
5. Falsey, A. R., Erdman, D., Anderson, L. J. & Walsh, E. E. Human metapneumovirus infections in young and elderly adults. J Infect Dis. 2003; 187: 785–790.
6. Afonso CL, Amarasinghe GK, Ba´nyai K, Ba`o Y, Basler CF, Bavari S, Bejerman N, et al. Taxonomy of the order Mononegavirales: update 2016. Arch Virol. 2016; 161:2351–2360.
7. Peret TC, Boivin G, Li Y, Couillard M, Humphrey C, Osterhaus AD, et al. Characterization of human metapneumoviruses isolated from patients in North America. J Infect Dis. 2002;185:1660–1663.
8. van den Hoogen BG, Herfst S, Sprong L, Cane PA, Forleo-Neto E, de Swart RL, et al. Antigenic and genetic variability of human metapneumoviruses. Emerg Infect

Dis 2004;10:658–66.

9. Huck B, Scharf G, Neumann-Haefelin D, Puppe W, Weigl J, Falcone V. Novel human metapneumovirus sublineage. Emerg Infect Dis. 2006;12:147–150.
10. Panda S, Nirmal KM, Lindomar P, Subrat K. Human metapneumovirus: review of an important respiratory pathogen. Int J Infect Dis. 2014;25:45-52.
11. Choudhary ML, Anand SP, Sonawane NS, Chadha MS. Development of real-time RT-PCR for detection of human metapneumovirus and genetic analysis of circulating strains (2009-2011) in Pune, India. Arch Virol. 2013; 159:217-225.
12. Zhang C, Du LN, Zhang ZY, Qin X, Yang X, et al. Detection and genetic diversity of human metapneumovirus in hospitalized children with acute respiratory infections in Southwest China. J Clin Microbiol. 2012; 50:2714–2719.
13. Bastien N, Ward D, Van Caeseele P, Brandt K, Lee SH, et al. Human metapneumovirus infection in the Canadian population. J Clin Microbiol.2003; 41: 4642–4646.
14. Carneiro BM, Yokosawa J, Arbiza J, Costa LF, Mirazo S, Nepomuceno LL, et al. Detection of all four human metapneumovirus subtypes in nasopharyngeal specimens from children with respiratory disease in Uberlandia, Brazil. Journal of medical virology. 2009; 81(10):1814–1818.
15. Galiano M, Videla C, Puch SS, Martinez A, Echavarria M, Carballal G (2004) Evidence of human metapneumovirus in children in Argentina. J Med Virol. 2004;72:299–303.
16. Mirazo S, Ruchansky D, Blanc A, Arbiza J. Serologic evidence of human metapneumovirus circulation in Uruguay. Mem Inst Oswaldo Cruz. 2005;100:715–718.

17. Pizzorno A, Masner M, Medici C, Sarachaga MJ, Rubio I, Mirazo S, Frabasile S, Arbiza J. Molecular detection and genetic variability of human metapneumovirus in Uruguay. J Med Virol. 2010;82:861–865.
18. Luchsinger V, Escobar C, Avendano LF. Detection of human metapneumovirus in children hospitalized for acute lower respiratory infection in Santiago, Chile. Rev Med Chil. 2005;133:1059–1064.
19. Garcia J, Sovero M, Kochel T, Laguna-Torres VA, Gamero ME, Gomez J, Sanchez F, Arango AE, Jaramillo S, Halsey ES. Human metapneumovirus strains circulating in Latin America. Arch Virol. 2012; 157:563–568.
20. Ulloa-Gutierrez R, Vargas-Jimenez F, Mora-Chavarria A, Umana MA, Calvo-Espinoza M, Alfaro-Bourrouet W. Human metapneumovirus in Costa Rica. Pediatr Infect Dis J. 2009;28:452–453.
21. Barbosa Ramirez J, Pulido Dominguez P, Rey Benito G, Mendez Rico J, Castellanos J, Páez Martinez A. Human respiratory syncytial virus and metapneumovirus in patients with acute respiratory infection in Colombia, 2000–2011. Rev Panam Salud Publica. 2014;36(2):101–9.
22. Schlaudecker EP, Heck JP, Macintyre ET et al. Etiology and Seasonality of Viral Respiratory Infections in Rural Honduran Children. Pediatr Infect Dis J 2012; 31:1113–1118.
23. McCracken JP, Arvelo W, Ortíz J, et al. Comparative epidemiology of human metapneumovirus- and respiratory syncytial virus-associated hospitalizations in Guatemala. Influenza and Other Respiratory Viruses. 2014;8(4):414-421.
24. Mackay IM, Bialasiewicz S, Waliuzzaman Z, Chidlow GR, Fegredo DC, Laingam S, et al. Use of the P gene to genotype human metapneumovirus identifies 4 viral

subtypes. 2004; Infect Dis 190:1913–1918.

25. Banerjee S, Sullender WM, Choudekar A, John C, Tyagi V, Fowler K, Lefkowitz EJ, Broor S. Detection and genetic diversity of human metapneumovirus in hospitalized children with acute respiratory infections in India. J Med Virol. 2011; 83:1799–1810.
26. Nei M, Kumar S. Molecular Evolution and Phylogenetics. Oxford University Press, New York. 2000.
27. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30:2725–2729.
28. Williams JV, Harris PA, Tollefson SJ, et al. Human Metapneumovirus and Lower Respiratory Tract Disease in Otherwise Healthy Infants and Children. The New England journal of medicine. 2004;350(5):443-450.
29. Lo Presti A, Cammarota R, Apostoli P, Cella E, Fiorentini S, Babakir-Mina M, et al. Genetic variability and circulation pattern of human metapneumovirus isolated in Italy over five epidemic seasons. The new microbiologica. 2011; 34(4):337–44.
30. Papenburg J, Carbonneau J, Isabel S, Bergeron MG, Williams JV, De Serres G, et al. Genetic diversity and molecular evolution of the major human metapneumovirus surface glycoproteins over a decade. Journal of clinical virology: the official publication of the Pan American Society for Clinical Virology. 2013; 58(3):541–547.

Figure Legends

Phylogenetic analysis performed with the Nucleoprotein (N) gene of HMPV Panamanian strains and reference sequences from identified genotypes. Phylogenetic tree was constructed using the Maximum Likelihood method based on the General Time Reversible model. Bootstrap statistical supports calculated from 2000 replicas are depicted at the nodes Evolutionary analyses were conducted in MEGA6. The sequence of the strain AF176590 from avian metapneumovirus was used as outgroup species. Nucleotide sequences from Panamanian strains were named as: strain code/PAN/collection year.

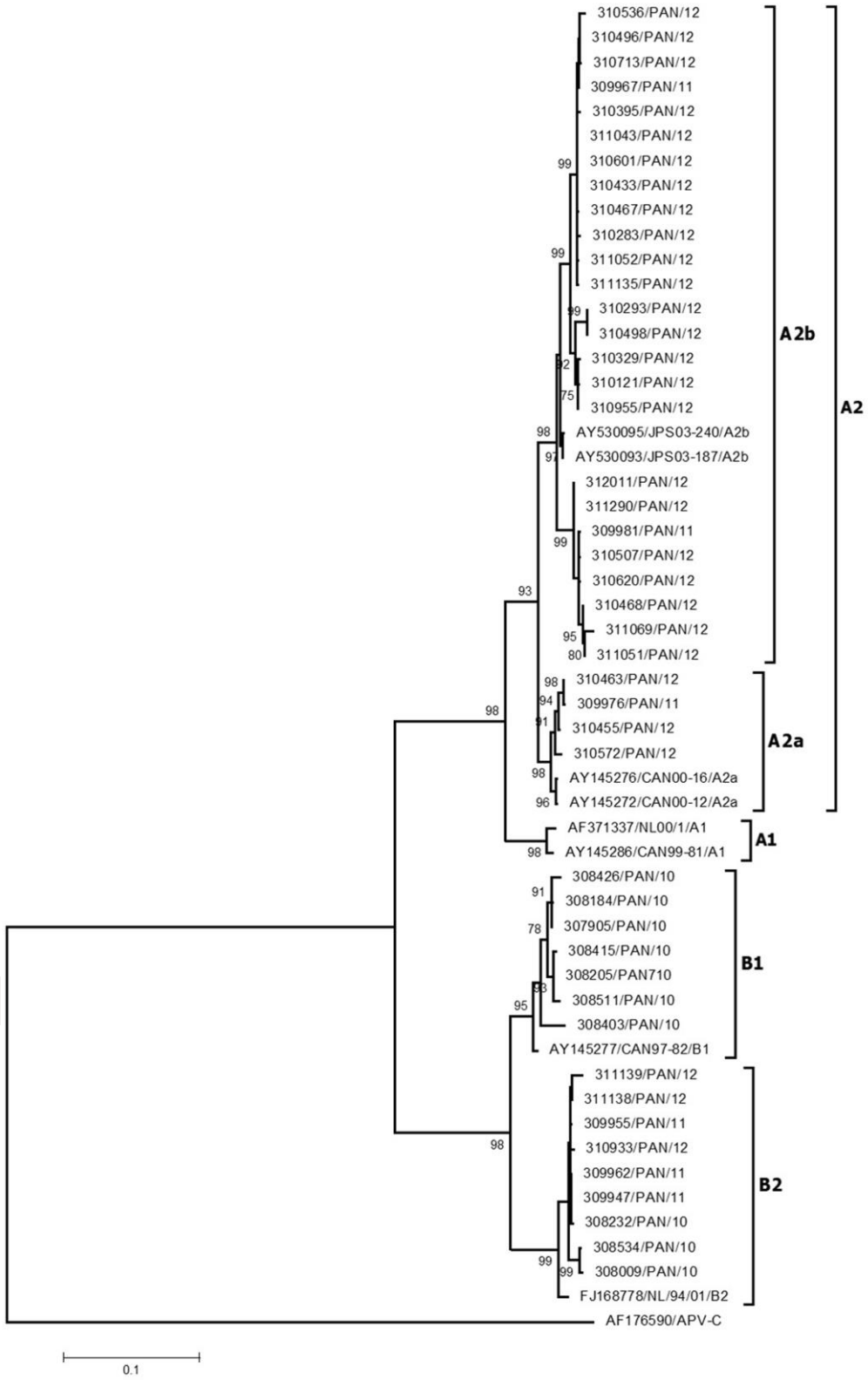


Figure 1

CONCLUSIONES Y PERSPECTIVAS

La variabilidad genética del VRSH fue demostrada en cepas de Panamá tanto para el grupo A como para el B. También se observó heterogenidad genética del MPVH al demostrar la circulación de 4 sublinajes.

El estudio de las cepas panameñas del VRSH-A mostró la circulación de 2 genotipos. Cuarenta y dos de las cepas analizadas, presentaron la inserción de 72 nucléotidos en la región C-terminal de la proteína G. Filogenéticamente, estas cepas pertenecen al nuevo genotipo ON1, descrito en 2010 a partir de cepas aisladas en Ontario (Canadá). Este genotipo se ha convertido en el predominante a nivel mundial, observándose como mayoritario en las distintas epidemias a partir de los años 2010-2011. El resto de las cepas (7), no presentaron esta inserción y mostraron tener similitud con el genotipo NA1.

El estudio antigénico realizado con cepas del VRSH-A, mediante el uso de anticuerpos monoclonales contra la proteína G, demostró que a pesar del complejo patrón de diversificación del VRSH-A observado en los últimos 50 años y la dominancia temporal de genotipos a lo largo del tiempo la variabilidad genética en el gen G podría no estar directamente relacionada con cambios antigénicos en la proteína G.

El estudio de las variantes circulantes de VRSH-B mostró el predominio absoluto del genotipo BA, en concordancia con los estudios reportados a nivel mundial. Se observó además la circulación de un nuevo genotipo, al que nombramos como “BA14”. El análisis de secuencias deducidas de aminoácidos de VRSH-B, mostró cambios previamente reportados y otros sólo encontrados en las cepas de Panamá.

El nuevo genotipo reportado en este estudio para VRSH-B (BA14), solo se detectó en cepas del 2008. Como perspectiva, nos planteamos analizar cepas previas a este año, con el fin de determinar el año de emergencia de este genotipo encontrado en Panamá y posteriores al 2012, para conocer posible re-emergencia de este genotipo.

El reemplazo y predominio de los genotipos que cuentan con la inserción de 60 (BA para VRSH-B) y 72 (ON1, para VRSH-A) nucléotidos en el gen que codifica para la proteína G fue observado en en la mayoría de las cepas de Panamá analizadas. Estos constantes cambios observados (inserciones, deleciones y susituciones aminoacídicas) a nivel mundial para el VRSH, hacen necesarios el seguimiento de las diferentes epidemias basado en estudios de variabilidad genética y antigénica.

Se demostró la circulación de cuatro sublinajes de MPVH mediante el análisis de secuencias parciales del gen N. Siendo este gen relativamente conservado, proponemos avanzar en el conocimoento de la variabilidad genética de este virus mediante el análisis del gen G.

REFERENCIAS BIBLIOGRÁFICAS

- Alvarez, R., K. S. Harrod, W. J. Shieh, S. Zaki, and R. A. Tripp. 2004. Human metapneumovirus persists in BALB/c mice despite the presence of neutralizing antibodies. JVirol 78:14003–14011.
- Arnott, A. S. et al . 2011. A Study of the Genetic Variability of Human Respiratory Syncytial Virus (RSV) in Cambodia Reveals the Existence of a New RSV Group B Genotype. J Clin Microbiol. 49:3504–3513.
- Afonso CL, Amarasinghe GK, Ba´nyai K, Ba`o Y, Basler CF, Bavari S, BejermanN, et al. Taxonomy of the order Mononegavirales: update 2016. ArchVirol 2016;161:2351–2360.
- Anderson LJ, Hierholzer JC, Tsou C, et al. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J Infect Dis. 1985;151:626–633.
- Baek, Y. H., et al., 2012. Prevalence and genetic characterization of respiratory syncytial virus (RSV) in hospitalized children in Korea. Arch Virol. 157:1039-1050.
- Beem M, Wright FH, Hamre D, Egerer R, and Oehme M. 1960. Association of the chimpanzee coryza agent with acute respiratory disease in children. NEJM; 263(11): 523-530.
- Bäyon-Auboyer, M. H., C. T. D. Arnauld, and N. Eterradossi. 2000. Nucleotide sequences of the F, L and G protein genes of two non-A/non-B avian pneumoviruses (APV) reveal a novel APV subgroup. J. Gen. Virol. 81:2723–2733.
- Boivin G, Abed Y, Pelletier G, Ruel L, Moisan D, Cote S, Peret TC, Erdman DD,Anderson LJ. Virological features and clinical manifestations associated with human metapneumovirus: a new paramyxovirus responsible for acute respiratory211 trac infections in all age groups. J Infect Dis. 2002; 186:1330–1334.
- Cane PA, 2001. Molecular epidemiology of respiratory syncytial virus. Rev Med Virol. 11(2):103-16.
- Carter SD, Dent KC, Atkins E, Foster TL, Verow M, Gorny P, Harris M, Hiscox JA, Ranson NA, Griffin S, Barr JN. 2010. Direct visualization of the small hydrophobic protein of human respiratory syncytial virus reveals the structural basis for membrane permeability. FEBS Lett. 2010; 584(13):2786–2790.
- Collins PL, 2013. Respiratory Syncytial Virus: Virology, Reverse Genetics, and Pathogenesis of Disease. Curr Top Microbiol Immunol. 372: 3–38.
- Chanock R, and Finberg L. 1957. Recovery from infants with respiratory illness of virus related to chimpanzee coryza gent (CCA) II. Epidemiologic aspects of infection in infants and young children. Am J Hyg; 66: 291-300.

- Cui G, Zhu R, Qian Y, et al. Genetic variation in attachment glycoprotein genes of human respiratory syncytial virus subgroups A and B in children in recent five consecutive years. PLoS ONE. 2013;8:1–13.
- Bembridge, G. P. et al., 2000. Respiratory syncytial virus infection of gene gun vaccinated mice induces Th2-driven pulmonary eosinophilia even in the absence of sensitisation to the fusion (F) or attachment (G) protein. Vaccine. 19:1038-46.
- Barbosa Ramirez J, Pulido Dominguez P, Rey Benito G, Mendez Rico J, Castellanos J, Páez Martinez A. 2014. Human respiratory syncytial virus and metapneumovirus in patients with acute respiratory infection in Colombia, 2000–2011. Rev Panam Salud Publica. 36(2):101–9.
- Beneri, C., C. C. Ginocchio, R. Manji, and S. Sood. 2009. Comparison of clinical features of pediatric respiratory syncytial virus and human metapneumovirus infections. Infect. Control Hosp. Epidemiol. 30:1240–1241.
- Biacchesi S. et al. 2004. Recombinant human Metapneumovirus lacking the small hydrophobic SH and/or attachment G glycoprotein: deletion of G yields a promising vaccine candidate. J Virol.78:12877–87.
- Biacchesi, S. et al . 2005. Infection of nonhuman primates with recombinant human metapneumovirus lacking the SH, G, or M2-2 protein categorizes each as a nonessential accessory protein and identifies vaccine candidates. J. Virol. 79:12608–12613.
- Blanc, A., A. Delfraro, S. Frabasile, and J. Arbiza. 2005. Genotypes of respiratory syncytial virus group B identified in Uruguay. Arch Virol. 150:603–609.
- Black RE et al., 2010. Global, regional, and national causes of child mortality in 2008: a systematic analysis. Lancet 2010; 375: 1969–87.
- Boeckh, M., V. Erard, D. Zerr, and J. Englund. 2005. Emerging viral infections after hematopoietic cell transplantation. Pediatr. Transplant. 9(Suppl. 7):48–54.
- Boivin, G., et al . 2003. Human metapneumovirus infections in hospitalized children. Emerg. Infect. Dis. 9:634–640.
- Bryce, J., C. Boschi-Pinto, K. Shibuya and R. E. Black. 2005. The WHO Child Health Epidemiology Reference Group. WHO estimates of the causes of death in children. Lancet 365:1147–1152.
- Buchholz U. J., Nagashima K., Murphy B. R and Collins P. L. 2006. Live vaccines for human metapneumovirus designed by reverse genetics. Expert Rev Vaccines.2006;5:695–706.
- Bukreyev A., L. et al . 2008. The secreted form of respiratory syncytial virus G glycoprotein helps the virus evade antibody-mediated restriction of replication by acting as an antigen decoy and through effects on Fc receptor-bearing leukocytes. J Virol. 82:12191–12204.
- Cane, P. A., B. G. van den Hoogen, S. Chakrabarti, C. D. Fegan, and A. D. Osterhaus. 2003. Human metapneumovirus in a haematopoietic stem cell transplant recipient with fatal lower respiratory tract disease. Bone Marrow Transplant. 31:309–310.

- Collins P. L and B. S. Graham. 2008. Viral and Host Factors in Human Respiratory Syncytial Virus Pathogenesis. J Virol. 82(5): 2040–2055
- Collins P. L. et al . 2007 Respiratory syncytial virus and metapneumovirus. Fields Virology. USALippincott Williams & Wilkins:1601–1646. •• Provides an in-depth description of RSV virology.
- Dapat, I. C., et al . 2010. New genotypes within respiratory syncytial virus group B genotype BA in Niigata, Japan. J Clin Microbiol. 48:3423–3427.
- Dowell, S. F. et al . 1996. Respiratory syncytial virus is an important cause of community-acquired lower respiratory infection among hospitalized adults. J Infect Dis. 174:456-62.
- Dutch R. E., R. N. Hagglund, M. A. Nagel, R. G. Paterson and R. A. Lamb. 2001. Paramyxovirus fusion (F) protein: a conformational change on cleavage activation. Virology. 2001; 281:138–150.
- Escobar, G. J., A. Ragins, S. X. Li, L. Prager, A. S. Masaquel, P. Kipnis. 2010. Recurrent wheezing in the third year of life among children born at 32 weeks' gestation or later: relationship to laboratoryconfirmed, medically attended infection with respiratory syncytial virus during the first year of life. Arch Pediatr Adolesc Med. 164(10):915–922.
- Eshaghi, A. et al . 2012. Genetic variability of human respiratory syncytial virus a strain circulating in Ontario: a novel genotype with a 72 nucleotide G gene duplication. Plos one. 7(3)e32807.
- Fearns, R., and P. L. Collins. 1999. Role of the M2-1 transcription antitermination protein of respiratory syncytial virus in sequential transcription. J. Virol. 73:5852–5864.
- Feldman, S. A., S. Audet and J. A. Beeler. 2000. The fusion glycoprotein of human respiratory syncytial virus facilitates virus attachment and infectivity via an interaction with cellular heparan sulfate. J. Virol. 74:6442–6447.
- Feuillet, F., B. Lina, M. Rosa-Calatrava and G. Boivin. 2012. Ten years of human metapneumovirus research. J Clin Virol. 53:97-105.
- Franquet, T., et al . 2005. Human metapneumovirus infection in hematopoietic stem cell transplant recipients: high-resolution computed tomography findings. J. Comput. Assist. Tomogr. 29:223–227.
- Fuentes S, Tran KC, Luthra P, Teng MN, He B. Function of the respiratory syncytial virus small hydrophobic protein. J Virol. 2007; 81(15):8361–8366.
- Galiano, M. C., et al., 2005. Intragroup antigenic diversity of human respiratory syncytial virus (group A) isolated in Argentina and Chile. J Med Virol. 77:311–316.
- Gan SW, Tan E, Lin X, Yu D, Wang J, Tan GM, Vararattanavech A, Yeo CY, Soon CH, Soong TW, Pervushin K, Torres J. The small hydrophobic protein of the human respiratory syncytial virus forms pentameric ion channels. J Biol Chem. 2012; 287(29):24671–24689.

- Garcia J, Sovero M, Kochel T, Laguna-Torres VA, Gamero ME, Gomez J, Sanchez F, Arango AE, Jaramillo S, Halsey ES. Human metapneumovirus strains circulating in Latin America. Arch Virol. 2012; 157:563–568.
- Graham, B. S. 2011. Biological challenges and technological opportunities for respiratory syncytial virus vaccine development. Immunol Rev. 239:149–166.
- Hall C. B. 2001. Respiratory syncytial virus and parainfluenza virus. N Engl J Med 344:1917–1928.
- Garcia-Beato R, Martinez I, Franci C, Real FX, Garcia-Barreno B, Melero JA. 1996. Host cell effect upon glycosylation and antigenicity of human respiratory syncytial virus G glycoprotein. Virology 221:301–309.
- Hallak, L. K., D. Spillmann, P. L. Collins and M. E. Peeples. 2000. Glycosaminoglycan sulfation requirements for respiratory syncytial virus infection. J Virol. 74:10508–10513.
- Hamelin, M. E., et al., 2005. Pathogenesis of human metapneumovirus lung infection in BALB/c mice and cotton rats. J. Virol. 79:8894–8903.
- Hara, M et al., 2008. Human Metapneumovirus infection in febril children with lower respiratory deseases in primary care settings in the Iroshima, Japan. J. Infect. Dis., 61:500-502.
- Hashem, M. and C. B. Hall. 2003. Respiratory syncytial virus in healthy adults: the cost of a cold. J Clin Virol 27:14-21.
- Henderson, J., T. N. Hilliard, A. Sherriff, D. Stalker, N. Al Shammari, H.M. Thomas. 2005. Hospitalization for RSV bronchiolitis before 12 months of age and subsequent asthma, atopy and wheeze: a longitudinal birth cohort study. Pediatr Allergy Immunol. 16(5):386–392.Herfst, S., et al . 2008. Low-pH-induced membrane fusion mediated by human metapneumovirus F protein is a rare, strain-dependent phenomenon. J. Virol. 82:8891–8895.
- Huck B. et al. 2006. Human metapneumovirus infection in a hematopoietic stem cell transplant recipient with relapsed multiple myeloma and rapidly progressing lung cancer. J Clin Microbiol. 44:2300–3.
- human metapneumovirus induces hypersensitivity to hMPV infection. Vaccine. 25:8518–28.
- Hurwitz, J. L. 2011. Respiratory syncytial virus vaccine development. Expert Rev Vaccines.10(10): 1415–1433.
- Karron, R. A. et al . 2005. Identification of a recombinant live attenuated respiratory syncytial virus vaccine candidate that is highly attenuated in infants. J Infect Dis. 191:1093-104..
- Kim, J-Y. and J. Chang. 2012. Need for a safe vaccine against respiratory syncytial virus infection. Korean J pediatr. 55(9):309-305.

- Kohlmann, R. et al . 2009. Protective efficacy and immunogenicity of an adenoviral vector vaccine encoding the codon-optimized F protein of respiratory syncytial virus. J Virol. 83:12601-10.
- Kuiken, T., et al . 2004. Experimental human metapneumovirus infection of cynomolgus macaques (Macaca fascicularis) results in virus replication in ciliated epithelial cells and pneumocytes with associated lesions throughout the respiratory tract. Am. J. Pathol. 164:1893–1900.
- Kurt-Jones E. A. et al . 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol.1:398–401.
- Lamb, R. A. 1993. Paramyxovirus fusion: a hypothesis for changes. Virology 197:1–11.
- Lamb, R. A. P., and G. D. Parks. 2007. Paramyxoviridae: the viruses and their replication. En D. M. Knipe et al . (ed.), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.p. 1449–1456.
- Lamb, R. A., and T. S. Jardetzky. 2007. Structural basis of viral invasion: lessons from paramyxovirus F. Curr. Opin. Struct. Biol. 17:427–436.
- Levine, A. M. and J. A. Whitsett. 2001. Pulmonary collectins and innate host defense of the lung. Microbes Infect. 3:161–166.
- Li, X. et al .1998. Protection against respiratory syncytial virus infection by DNA immunization. J Exp Med. 188:681-8.
- Liu, L., and N. Bastien, Y. Li. 2007. Intracellular processing, glycosylation, and cell surface expression of human metapneumovirus attachment glycoprotein. J. Virol. 81:13435–13443.
- McCracken JP, Arvelo W, Ortíz J, et al. Comparative epidemiology of human metapneumovirus- and respiratory syncytial virus-associated hospitalizations in Guatemala. Influenza and Other Respiratory Viruses. 2014;8(4):414-421.24.
- Mackay IM, Bialasiewicz S, Waliuzzaman Z, Chidlow GR, Fegredo DC, Laingam S, et al. Use of the P gene to genotype human metapneumovirus identifies 4 viral subtypes. 2004; Infect Dis 190:1913–1918.
- Morris JA, Blount RE, and Savage RE. 1956. Recovery of cytopathogenic agent from chimpanzee with coryza. Proc Soc Exp Biol & Med; 92:544-549.
- Mufson MA, Orvell C, Rafnar B, Norrby E. 1985. Two distinct subtypes of human respiratory syncytial virus. J Gen Virol 66:2111–2124.
- Murphy, B. R., A. V. Sotnikov, L. A. Lawrence, S. M. Banks, G. A. Prince. 1990. Enhanced pulmonary histopathology is observed in cotton rats immunized with formalin-inactivated respiratory syncytial virus (RSV) or purified F glycoprotein and challenged with RSV 3-6 months after immunization. Vaccine. 8:497-502.
- Peret TC, Boivin G, Li Y, Couillard M, Humphrey C, Osterhaus AD, et al. Characterization of human metapneumoviruses isolated from patients in North America. J Infect Dis. 2002;185:1660–1663.

- Palomo C, Cane PA, Melero JA. 2000. Evaluation of the antibody specificities of human convalescent-phase sera against the attachment (G) protein of human respiratory syncytial virus: influence of strain variation and carbohydrate side chains. J Med Virol 60:468–474.
- Pelletier, G., P. Dery, Y. Abed, and G. Boivin. 2002. Respiratory tract reinfections by the new human metapneumovirus in an immunocompromised child. Emerg. Infect. Dis. 8:976–978.
- Peret, T. C., C. B. Hall, K. C. Schnabel, J. A. Golub, and L. J. Anderson. 1998. Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. J Gen Virol. 79(Pt.9):2221–2229.
- Peret, T. C. et al., 2000. Circulation patterns of group A and B human respiratory syncytial virus genotypes in 5 communities in North America. J Infect Dis. 181:1891–1896.
- Roberts S. R., D. Lichtenstein, L. A. Ball and G. W. Wertz. 1994. The membrane-associated and secreted forms of the respiratory syncytial virus attachment glycoprotein G are synthesized from alternative initiation codons. J Virol. 68:4538–4546.
- Rudan I, Boschi-Pinto C, Zrinka Biloglav Z, Kim Mulhollandd K, Campbelle H. 2008. Epidemiology and etiology of childhood pneumonia. Bulletin of the World Health Organization; 86:408–416.
- Russell, C. J., and L. E. Luque. 2006. The structural basis of paramyxovirus invasion. Trends Microbiol. 14:243–246.
- Schildgen, V., et al . 2011. Human Metapneumovirus: lessons learned over the first decade. Clin Microbiol Rev. 24(4):734-54.
- Schowalter, R. M., S. E. Smith, and R. E. Dutch. 2006. Characterization of human metapneumovirus F protein-promoted membrane fusion: critical roles for proteolytic processing and low pH. J. Virol. 80:10931–10941.
- Stegmann T, Kamphuis T, Meijerhof T, et al. Lipopeptide-adjuvanted respiratory syncytial virus virosomes: a safe and immunogenic non-replicating vaccine formulation. Vaccine. 2010; 28:5543–5550.
- Sigurs, N. et al., 2005. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. Am J Respir Crit Care Med.171(2):137–141.
- Skiadopoulos M. H. et al. 2004. The two major human metapneumovirus genetic lineages are highly related antigenically, and the fusion (F) protein is a major contributor
- Stein, R. T. et al.,. 1999. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. Lancet. 1999; 354(9178):541–545.
- Sugrue R. J., C. Brown, G. Brown, J. Aitken and R. H. McL. 2001. Furin cleavage of the respiratory syncytial virus fusion protein is not a requirement for its transport to the surface of virus-infected cells. J Gen Virol. 2001; 82:1375–1386.

- Shobugawa Y, Saito R, Sano Y, et al. Emerging genotypes of human respiratory syncytial virus subgroup A among patients in Japan. J Clin Microbiol. 2009;47:2475–2482.
- de Swart, R. L., et al. 2007. Immunization of macaques with formalininactivated human metapneumovirus induces hypersensitivity to hMPV infection. Vaccine 25:8518–8528.
- Techaarpornkul, S. and P. L. Collins, M. E. 2002. Peeples. Respiratory syncytial virus with the fusion protein as its only viral glycoprotein is less dependent on cellular glycosaminoglycans for attachment than complete virus. Virology. 294:296–304.
- Teng, M. N., S. S. Whitehead, and P. L. Collins. 2001. Contribution of the respiratory syncytial virus G glycoprotein and its secreted and membranebound forms to virus replication in vitro and in vivo. Virology 289:283–296.
- Thammawat, S., T. A. Sadlon, P. G. Hallsworth, and D. L. Gordon. 2008. Role of cellular glycosaminoglycans and charged regions of viral G protein in human metapneumovirus infection. J. Virol. 82:11767–11774.
- Trento, A., et al . 2006. Natural history of human respiratory syncytial virus inferred from phylogenetic analysis of the attachment (G) glycoprotein with a 60-nucleotide duplication. J Virol 80:975–984.
- Tripp, R. A. et al .2001. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. Nat Immunol. 2:732–738.
- van den Hoogen B. G. et al. 2004. Antigenic and genetic variability of human metapneumoviruses. Emerg Infect Dis. 10:658–66.
- van den Hoogen, B. G, et al . 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med. 7(6):719-24.
- van den Hoogen, B. G. 2007. Respiratory tract infection due to human metapneumovirus among elderly patients. Clin. Infect. Dis. 44:1159–1160.
- van den Hoogen, B. G., D. M. Osterhaus, and R. A. Fouchier. 2004. Clinical impact and diagnosis of human metapneumovirus infection. Pediatr. Infect. Dis. J. 23:S25–S32.
- van den Hoogen, B. G., et al . 2003. Prevalence and clinical symptoms of human metapneumovirus infection in hospitalized patients. J. Infect. Dis. 188:1571–1577.
- van den Hoogen, B. G., et al . 2003. Prevalence and clinical symptoms of human metapneumovirus infection in hospitalized patients. J. Infect. Dis. 188:1571–1577.
- van den Hoogen, B. G., T. M. Bestebroer, A. D. Osterhaus, and R. A. Fouchier. 2002. Analysis of the genomic sequence of a human metapneumovirus. Virology 295:119–132.
- van Woensel, J. B., A. P. Bos, R. Lutter, J. W. Rossen, and R. Schuurman. 2006. Absence of human metapneumovirus co-infection in cases of severe respiratory syncytial virus infection. Pediatr. Pulmonol. 41:872–874.
- Venter, M., S. A. Madhi, C. T. Tiemessen, and B. D. Schoub. 2001. Genetic diversity and molecular epidemiology of respiratory syncytial virus over four consecutive

seasons in South Africa: identification of new subgroup A and B genotypes. J. Gen. Virol. 82:2117–2124.

- Vuola J. M., et al. 2005. Differential immunogenicity of various heterologous prime-boost vaccine regimens using DNA and viral vectors in healthy volunteers. J Immunol. 174:449–55.
- Williams, J. V., et al . 2004. Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. N. Engl. J. Med. 350:443–450.
- Williams, J. V., et al . 2005. A prospective study comparing human metapneumovirus with other respiratory viruses in adults with hematologic malignancies and respiratory tract infections. J. Infect. Dis. 192:1061–1065.
- Williams, J. V., et al . 2005. Human metapneumovirus infection plays an etiologic role in acute asthma exacerbations requiring hospitalization in adults. J. Infect. Dis. 192:1149–1153.
- Williams, J. V., S. J. Tollefson, J. E. Johnson, and J. E. Crowe, Jr. 2005. The cotton rat (Sigmodon hispidus) is a permissive small animal model of human metapneumovirus infection, pathogenesis, and protective immunity. J. Virol. 79:10944–10951.
- Wu, P. and T. V Hartert. 2011. Evidence for a causal relationship between respiratory syncytial virus infection and asthma. Expert Rev Anti Infect Ther. 9(9): 731–745.
- Wyde, P. R., S. N. Chetty, A. M. Jewell, S. L. Schoonover, and P. A. Piedra. 2005. Development of a cotton rat-human metapneumovirus (HMPV) model for identifying and evaluating potential HMPV antivirals and vaccines. Antiviral Res. 66:57–66.
- Choudhary ML, Anand SP, Sonawane NS, Chadha MS. Development of real-time
- RT-PCR for detection of human metapneumovirus and genetic analysis of circulating strains (2009-2011) in Pune, India. Arch Virol. 2013; 159:217-225.
- Zhang C, Du LN, Zhang ZY, Qin X, Yang X, et al. Detection and genetic diversity of human metapneumovirus in hospitalized children with acute respiratory infections in Southwest China. J Clin Microbiol. 2012; 50:2714–2719.
- Galiano M, Videla C, Puch SS, Martinez A, Echavarria M, Carballal G. 2004. Evidence of human metapneumovirus in children in Argentina. J Med Virol. 72:299–303.
- Mirazo S, Ruchansky D, Blanc A, Arbiza J. Serologic evidence of human metapneumovirus circulation in Uruguay. Mem Inst Oswaldo Cruz. 2005;100:715–718.
- Pizzorno A, Masner M, Medici C, Sarachaga MJ, Rubio I, Mirazo S, Frabasile S, Arbiza J. Molecular detection and genetic variability of human metapneumovirus in Uruguay. J Med Virol. 2010;82:861–865.
- Luchsinger V, Escobar C, Avendano LF. Detection of human metapneumovirus in children hospitalized for acute lower respiratory infection in Santiago, Chile. Rev Med Chil. 2005;133:1059–1064.

- Garcia J, Sovero M, Kochel T, Laguna-Torres VA, Gamero ME, Gomez J, Sanchez F, Arango AE, Jaramillo S, Halsey ES. Human metapneumovirus strains circulating in Latin America. Arch Virol. 2012; 157:563–568.
- Ulloa-Gutierrez R, Vargas-Jimenez F, Mora-Chavarria A, Umana MA, Calvo-Espinoza M, Alfaro-Bourrouet W. Human metapneumovirus in Costa Rica. Pediatr Infect Dis J. 2009;28:452–453.
- Schlaudecker EP, Heck JP, Macintyre ET et al. Etiology and Seasonality of Viral Respiratory Infections in Rural Honduran Children. Pediatr Infect Dis J 2012; 31:1113–1118.
- Lo Presti A, Cammarota R, Apostoli P, Cella E, Fiorentini S, Babakir-Mina M, et al. 2011. Genetic variability and circulation pattern of human metapneumovirus isolated in Italy over five epidemic seasons. The new microbiologica. 34(4):337–44.
- Weber MW, Mulholland EK, Greenwood BM. Respiratory syncytial virus infection in tropical and developing countries. Trop Med Int Health. 1998;3:268–280. Pretorius MA, Van Niekerk S, Tempia S, et al. Replacement and positive evolution of subtype a and b respiratory syncytial virus g-protein genotypes from 1997-2012 in South Africa. J Infect Dis. 2013;208:S227–S237.
- Trento A, Ábrego L, Rodriguez-Fernandez R, et al. Conservation of G protein epitopes in respiratory syncytial virus (Group A) despite broad genetic diversity: is antibody selection involved in virus evolution? J Virol. 2015;89:7776–7785.
- Trento A, Casas I, Calderón A, et al. Ten years of global evolution of the human respiratory syncytial virus BA genotype with a 60-nucleotide duplication in the G protein gene. J Virol. 2010;84:7500–7512.
- Gimferrer L, Andrés C, Campins M. Circulation of a novel human respiratory syncytial virus group B genotype during the 2014-2015 season in Catalonia (Spain). Clin Microbiol Infect. 2015;448:11–14.
- Khor CS, Sam IC, Hooi PS, Chan YF. Displacement of predominant respiratory syncytial virus genotypes in Malaysia between 1989 and 2011. Infect Genet Evol. 2013;14:357–360.
- van Niekerk S, Venter M. Replacement of previously circulating respiratory syncytial virus subtype B Strains with the BA genotype in South Africa. J Virol. 2011;85:8789–8797.
- Zlateva KT, Lemey P, Moës E, Van Ranst M, Moe E. Genetic variability and molecular evolution of the human respiratory syncytial virus subgroup B attachment G protein genetic variability and molecular evolution of the human respiratory syncytial virus subgroup b attachment g protein. J Virol. 2005;79:9157.
- Cuevas, L. E., et al. 2003. Human metapneumovirus and respiratory syncytial virus, Brazil. Emerg. Infect. Dis. 9:1626–1628.

- Peiris, J. S., et al. 2003. Children with respiratory disease associated with metapneumovirus in Hong Kong. Emerg. Infect. Dis. 9:628–633.
- Whitehead SS, Bukreyev A, Teng MN, Firestone CY, St Claire M, Elkins WR, Collins PL, Murphy BR. Recombinant respiratory syncytial virus bearing a deletion of either the NS2 or SH gene is attenuated in chimpanzees. J Virol. 1999; 73(4):3438–3442.