UNIVERSIDAD DE COSTA RICA
SISTEMA DE ESTUDIOS DE POSGRADO

MURCIÉLAGOS QUE HABITAN EN CASAS: ¿POSIBLES INVOLUCRADOS EN EL CICLO DE TRANSMISIÓN DEL DENGUE?

Tesis sometida a la consideración de la Comisión del Programa de Estudios de Posgrado en Biología para optar al grado y título de Maestría Académica en Biología con énfasis en Genética y Biología Molecular

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A mis papás, como todo, como siempre.

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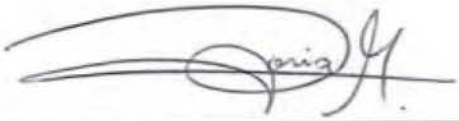

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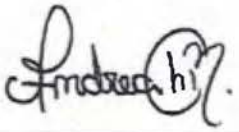

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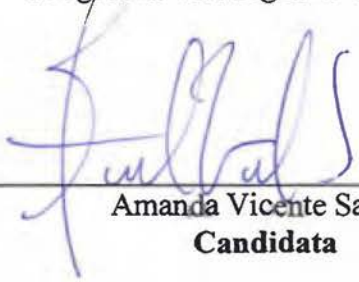

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Resumen

El dengue es la enfermedad humana transmitida por un vector más importante en todo el mundo. Se ha reportado constantemente la importancia de la vida silvestre como reservorios u hospederos en enfermedades infecciosas (re)emergentes. Recientemente, algunos estudios han demostrado la presencia de ácidos nucleicos del virus de dengue (DENV) y anticuerpos neutralizantes contra DENV en fauna neotropical, incluyendo murciélagos. Es posible que algunas especies de murciélagos puedan ser susceptibles a la infección por DENV. Esta investigación tiene como objetivo dilucidar el papel de los murciélagos que habitan en casas en el ciclo de transmisión del virus del dengue. Tomamos muestras de murciélagos en regiones de alta y baja incidencia del dengue durante las estaciones seca y lluviosa en Costa Rica, donde el dengue es considerada una enfermedad hiper endémica con la co-circulación de los cuatro serotipos. Capturamos 318 murciélagos de 11 especies diferentes utilizando redes de niebla en 29 casas en las que los seres humanos y los murciélagos cohabitan. Las necropsias se realizaron en 205 murciélagos para analizar la posible replicación del virus en el corazón, pulmón, bazo, hígado, riñón y cerebro. No encontramos la presencia de ARN de DENV en ningún órgano. Colectamos muestras de sangre de todos los especímenes y las analizamos mediante neutralización y PCR. Obtuvimos una seroprevalencia del 22% (52/241). El ARN de DENV lo detectamos en el 8,8% de los murciélagos muestreados (28/318). De estos 28 murciélagos, analizamos 11 muestras de intestino por PCR y dos individuos fueron positivos por ARN de DENV, correspondiente al mismo serotipo del dengue detectado en la sangre. Intentamos aislar el virus sin obtener resultados exitosos. Además, analizamos la carga viral mediante qRT-PCR y obtuvimos concentraciones muy baja de virus en muestras de sangre positivas, lo que sugiere una concentración de virus por debajo del umbral mínimo necesario para la infección por la alimentación de mosquito. Al mismo tiempo, colectamos 651 mosquitos mediante trampas EVS-CO_2 y los analizamos para la detección de DENV y citocromo b de murciélago en el contenido estomacal. Sólo tres pooles de mosquitos fueron positivos para el dengue y todos los mosquitos fueron negativos para citocromo b de murciélago. Nuestros resultados sugieren la presencia accidental de ácidos nucleicos del virus del dengue en murciélagos y que su infección da lugar posiblemente a partir de la ingestión oral de mosquitos infectados. Los análisis filogenéticos sugieren incluso un caso de derrame epidemiológico de brotes de dengue en humanos a murciélagos. Por lo tanto, llegamos a la conclusión de que los murciélagos no parecen sostener la amplificación DENV y no tienen un papel como reservorios, sino que representan un callejón sin salida epidemiológica para este virus.

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Capítulo 1

Lista de Abreviaturas

AEC	3-amino-9-cabazol etílico
ARN	Ácido ribonucleico
BLAST	Herramienta de búsqueda de alineamiento básico de nucléotidos
C/prM	Genes de cápside y premembrana
C6/36	Células de cultivo de *Aedes albopictus*
cDNA	Ácido desoxirribonicleico copia
CICUA	Comité Institucional para el Cuido y Uso de los Animales
CMC	Carboximetilcelulosa
CO_2	Dióxido de carbono
COI	Subunidad I de la oxidasa del citocromo mitocondrial
CPE	Efecto citopático
Cyt b	Citocromo b
DENV	Virus del Dengue
DENV-1	Serotipo 1 del Virus del Dengue
DENV-2	Serotipo 2 del Virus del Dengue
DENV-3	Serotipo 3 del Virus del Dengue
DENV-4	Serotipo 4 del Virus del Dengue
DHF	Fiebre de dengue hemorrágico
DNA	Ácido desoxirribonucleico
DSS	Síndrome de choque por dengue
ELISA	Ensayo por inmunoadsorción ligado a enzimas
EVS-CO_2	Trampa de control de vectores de encefalitis con dióxido de carbono como atrayente
FBS	Suero fetal bovino
GLMM	Binomial Modelo lineal generalizado mixto binomial
H_2O_2	Peróxido de hidrógeno
JEV	Virus de la Encefalitis Japonesa
K2 + G	Modelo de dos párametros de Kimura con distribución gamma

MEM	Medio mínimo esencial
ML	Máxima verosimilitud
NS1	Proteína no estructural 1
OMS	Organización Mundial para la Salud
PCR	Reacción en cadena de la polimerasa
PBS	Tampón fosfato salino
PFU	Unidades formadoras de placas
prM/E	Genes premembrana y de envoltura
PRNT	Prueba de neutralización por reducción de placas
qRT-PCR cuantitativo	Transcripción reversa de la reacción en cadena de la polimerasa
RT-PCR	Transcripción reversa de la reacción en cadena de la polimerasa
SLEV	Virus de la Encefalitis de San Luis
UV light	Luz ultra violeta
WHO	Organización Mundial para la Salud
WNV	Virus del Oeste del Nilo
YFV	Virus de la Fiebre Amarrilla

"The baby bat
Screamed out in fright,
'Turn on the dark,
I'm afraid of the light."

A Light in the Attic
Shel Silverstein 1981

Prefacio

El Virus del Dengue (DENV) es el agente etiológico de la fiebre del dengue y sus cuadros graves en seres humanos como por ejemplo el dengue hemorrágico o síndrome de choque. Es un virus de ARN de cadena simple lineal de polaridad positiva del género Flavivirus y familia Flaviviridae (Murrell *et al.* 2011). El DENV presenta cuatro serotipos antigénicamente distintos pero genéticamente relacionados (DENV-1 a DENV-4). El DENV pertenece al grupo de los arbovirus, del inglés 'arthropod-borne virus'. Esto es un término ecológico y está relacionado a su dependencia de un artrópodo como vector, en este caso, de mosquitos del género *Aedes* (Gubler 2002). Como tal, la distribución geográfica del agente viral está restringida a los parámetros ecológicos que gobiernan su ciclo de transmisión. Factores limitantes como temperatura, precipitación y patrones de vegetación influyen en la distribución de los vectores y del hospedero o reservorio vertebrado requerido para el mantenimiento del ciclo viral (Gubler 2001).

En la actualidad, el dengue tiene una amplia distribución mundial, encontrándose en todas las zonas tropicales incluyendo a Costa Rica (Gubler 2001). La transmisión del DENV ocurre a lo largo de todo el año en los países tropicales donde la enfermedad es endémica; sin embargo, existe un patrón cíclico distintivo asociado a las temporadas de lluvia (Feng and Velasco-Hernández 1997). En América los picos de transmisión suceden en las temporadas donde la precipitación es mayor y las temperaturas son más altas (Feng and Velasco-Hernández 1997). En los últimos años, la transmisión del DENV entre seres humanos se ha intensificado. Se encuentran cerca de 2500 millones de personas en riesgo de infección en el mundo y la Organización Mundial de la Salud (OMS) estima que hay alrededor de 50 a 100 millones de casos por año (OMS and TDR 2009). Sin embargo, un estudio utilizando datos de ocurrencias y métodos cartográficos estima que se dan 390 millones de infecciones de dengue por año, de los cuales 96 millones se manifiestan aparentemente (cualquier síntoma asociado pero independiente del nivel de severidad de la enfermedad) (Bhatt *et al.* 2013).

El DENV tiene una estrecha relación con humanos que se ha intensificado en las últimas décadas debido a condiciones ecológicas alteradas, así como al comportamiento humano (comercio global, urbanización, grandes movimientos poblacionales) y programas insostenibles de control de vectores (Feng and Velasco-Hernández 1997, Gubler 2001, Troyo *et al.* 2006, Schneider *et al.* 2009, Weaver and Vasilakis 2009). Medidas de control de vectores son particularmente importantes para la prevención del dengue, debido a la falta de la vacuna aprobada para uso clínico y de la terapia antiviral específica contra dengue. Por más de 60 años se han realizado estudios para la elaboración de la vacuna, la cual debe ser rentable e inducir una respuesta inmune duradera y simultánea contra los cuatro serotipos de dengue. Aunque todavía no se cuenta con una, el candidato que ha avanzado más en estudios clínicos es la vacuna de Sanofi Pasteur CYD-TDV, aunque en la Fase III ha tenido una eficacia subóptima (Screaton *et al.* 2015).

La transmisión del DENV se presenta en dos ciclos distintos: (i) el ciclo endémico/epidémico o urbano que involucra al humano como hospedero y la transmisión por *Ae. aegypti*, con *Ae. albopictus* y otros mosquitos *Aedes* sirviendo como vectores secundarios; y (ii) un ciclo zoonótico o selvático en hábitats boscosos de África Central, África del Este y del sureste asiático, involucrando a los primates no humanos como huéspedes reservorios y varios mosquitos de dosel del género *Aedes* (Feng and Velasco-Hernández 1997, Weaver and Vasilakis 2009). Los ciclos de transmisión selvática han sido poco estudiados. Sin embargo, estudios filogenéticos sugieren que la forma selvática de este virus es ancestral, es decir, el origen del DENV urbano podría ser selvático (Weaver and Vasilakis 2009). Además, estudios entomológicos concuerdan con este hallazgo al proponer que el vector enlace entre el ciclo selvático y comunidades humanas localizadas en o cerca de los bosques africanos y asiáticos pudo ser *Ae. furcifer*, un mosquito de bosque con fácil dispersión en hábitats peridomésticos (Weaver and Vasilakis 2009). Estudios sugieren que debido a presiones selectivas que pudieron llevar a cambios genéticos en el patógeno, se pudo haber dado la adaptación del virus a nuevos vectores y hospederos vertebrados (Gubler 2001, Weaver and Vasilakis 2009). Ya que *Ae. aegypti* no existía en Asia y Oceanía antes del establecimiento del comercio

por barcos, *Ae. albopictus* fue probablemente el vector original de humanos. El papel de *Ae. aegypti* como vector pudo haber surgido solo durante los últimos siglos, cuando el comercio vía buques distribuyó al mosquito en los trópicos (Weaver and Vasilakis 2009).

En el Nuevo Mundo, a diferencia del Viejo Mundo, se cree que la infección del dengue está ausente en la fauna silvestre ya que el ciclo de transmisión viral introducido fue el endémico, con la aparición de su vector *Ae. aegypti* en el Neotrópico (Weaver and Vasilakis 2009). Sin embargo, varios estudios han documentado la presencia ácidos nucleicos del virus o de anticuerpos neutralizantes en mamíferos de vida silvestre en países del Nuevo Mundo (Platt *et al.* 2000, de Thoisy *et al.* 2004, 2009, Aguilar-Setién *et al.* 2008, Lavergne *et al.* 2009, Sotomayor-Bonilla *et al.* 2014). No obstante, se desconocen cuáles podrían ser los posibles mosquitos vectores en el bosque, ya que *Ae. aegypti* suele estar asociado a sitios urbanos (Lavergne *et al.* 2009).

Platt *et al.* (2000) realizaron un estudio en Costa Rica y Ecuador en donde, mediante la prueba de neutralización por reducción de placas (PRNT, por sus siglas en inglés), encontraron anticuerpos contra DENV en 12 de 53 y 3 de 10 murciélagos, respectivamente para cada país. Además de encontrar una alta seroprevalencia del virus en los murciélagos, observaron que *Ae. aegypti* se alimentaba de murciélagos en el laboratorio, por lo que plantearon la posibilidad de que estos mamíferos tuvieran un papel en el ciclo del virus y que posiblemente fueran reservorios del DENV. Sin embargo, Scott (2001) hace un llamado a la prudencia al analizar los resultados obtenidos, dada la posibilidad de falsos positivos por reacciones cruzadas con otros flavivirus y sugiere realizar más estudios al respecto.

Encontrar la presencia de anticuerpos contra un virus en mamíferos, indica que el animal ha estado en contacto con el mismo, pero no nos indica si hay infección activa o alguna presentación de manifestaciones clínicas. Sin embargo, en el caso del DENV, que presenta reacción cruzada con otros flavivirus, se requiere de pruebas adicionales que nos indiquen el virus específico al cual se expuso el individuo (Mackenzie *et al.* 2004, Calisher *et al.* 2008, Peeling *et al.* 2010). Esto es de especial importancia en

murciélagos, ya que algunas especies han demostrado ser susceptibles a distintos flavivirus en condiciones naturales y de laboratorio (Wong *et al.* 2007). Estudios han demostrado la detección molecular de antígenos, así como el aislamiento viral de algunos flavivirus, como es el caso del Virus del Oeste del Nilo (WNV), de la Encefalitis de San Luis (SLEV) y de la Encefalitis Japonesa (JEV) (Davis *et al.* 2005, Wong *et al.* 2007, Cui *et al.* 2008, Wang *et al.* 2009). Adicionalmente, se han encontrado algunos flavivirus solamente en murciélagos, como el Virus de Río Bravo, el Virus de Yokose y el Virus de la leucoencefalitis de Montana Myotis (Tajima *et al.* 2005, Calisher *et al.* 2006, Wong *et al.* 2007).

de Thoisy *et al.* (2004) examinaron la presencia de anticuerpos contra DENV, mediante pruebas de seroneutralización con sueros de especies de mamíferos silvestres capturados en bosques vírgenes en la Guyana Francesa. Encontraron anticuerpos neutralizantes contra DENV-2 en armadillos, puercoespines, zarigüeyas, guatusas y cabros de monte. Los autores propusieron que los animales silvestres pueden estar expuestos a DENV; y que, aunque su papel en el ciclo de trasmisión no se conoce, podrían tener una función como reservorios temporales con transmisión por *Ae. aegypti* en el bosque o por otros posibles vectores.

Aguilar-Setién *et al.* (2008a) realizaron un estudio en zonas endémicas de alta incidencia de dengue en seres humanos en el Pacífico de México. Mediante ELISA, RT-PCR (transcripción reversa de la reacción en cadena de la polimerasa) y la presencia de la proteína viral DENV NS1 (producida durante la fase de infección aguda y que está correlacionada con la replicación viral), encontraron cuatro de diecinueve especies de murciélagos positivas para DENV-2. Sin embargo, ellos citan que la presencia de DENV en murciélagos no confirma una infección activa del virus en los individuos capturados. Concluyeron que para justificar si el murciélago es susceptible a la infección con el virus, es necesario aislarlo del murciélago o realizarle inoculaciones controladas en el laboratorio. Detectar antígenos o ácidos nucleicos virales podría dar una evidencia de replicación viral y el aislamiento del virus indicaría que el animal sí se infectó activamente. Aun así, aislar el virus no indica qué papel juega el mamífero en el ciclo natural de ese virus (Calisher *et al.* 2008). Considerando los datos obtenidos, sugirieron

que aunque no descartan que los murciélagos actúen como reservorios amplificadores del virus, es posible que puedan cumplir un papel de conservar al DENV en la naturaleza.

Otros estudios realizados en la Guyana Francesa utilizando técnicas de RT-PCR, detectaron los cuatro serotipos en muestras de hígado y/o suero de 92 mamíferos silvestres (Lavergne *et al.* 2009, de Thoisy *et al.* 2009). Mediante análisis de secuencias determinaron que los serotipos DENV-1, DENV-3 y DENV-4 eran distintos a los que circulaban en humanos en ese momento en la misma área geográfica (Lavergne *et al.* 2009, de Thoisy *et al.* 2009). Por otro lado, los análisis para DENV-2 encontrado en los mamíferos silvestres demostraron que algunas cepas eran divergentes de cepas concurrentes de humanos, pero otras eran idénticas. Esto sugiere que los mamíferos neotropicales que viven en áreas periurbanas pueden encontrarse infectados por cepas del DENV que circulan en humanos, y que bajo la presión de un evento epidémico fuerte, las cepas urbanas podrían introducirse al bosque e infectar parte de la fauna. Debido a esto parece ser de gran importancia considerar a las especies silvestres no solo como reservorios potenciales para DENV, sino también como posibles hospederos, sensibles a la infección por los agentes infecciosos provenientes de humanos (de Thoisy *et al.* 2009). Éstas especies de mamíferos pueden ser un "callejón sin salida" desde el punto de vista epidemiológico, o pueden jugar un papel en mantener el virus en períodos inter epidémicos. Sin embargo, se requiere mayor investigación para determinar si estos animales son hospederos accidentales o potenciales reservorios del virus (de Thoisy *et al.* 2009).

La evidencia de estos estudios sugiere que una gran cantidad de casos humanos de dengue en un lugar y tiempo determinado, puede producir un desbordamiento infeccioso ("spillover") a otros mamíferos, o que exista un posible ciclo viral enzoótico que involucre mamíferos silvestres (Lavergne *et al.* 2009, de Thoisy *et al.* 2009). La pregunta que si los mamíferos pueden mantener el DENV en un ciclo selvático y si juegan un papel en el mantenimiento de la enfermedad en poblaciones humanas, todavía no ha sido respondida.

Otra investigación en México hizo un estudio serológico del WNV, SLEV y DENV en los murciélagos; y encontraron anticuerpos contra flavivirus en el 19% de los murciélagos, donde los títulos mayores de PRNT eran positivos para DENV-2 o DENV-4, sin embargo todos los títulos eran relativamente bajos, y es posible que los murciélagos habrían sido infectados por otros flavivirus no incluidos en el análisis (Machain-Williams *et al.* 2013). Dos estudios recientes han puesto a prueba la susceptibilidad de los murciélagos a la infección por DENV en condiciones de laboratorio. Perea-Martínez *et al.* (2013) demostraron que después de la inoculación intraperitoneal de DENV-2 en *Artibeus intermedius* (*A. lituratus*), el virus se replica poco, lo que sugiere que no son hospederos competentes para este virus. Además, Cabrera-Romo *et al.* (2014) inocularon *Artibeus jamaicensis* con DENV-1 y DENV-4 utilizando diferentes vías: por vía subcutánea, por vía intraperitoneal y por la picadura de un *Ae. aegypti* infectado. En algunos casos encontraron ARN de DENV (6/22) en bazo y NS1 (8/22) en el suero de los murciélagos, pero en concentraciones bajas y no fueron capaces de reproducir sus resultados. Los autores concluyen que los murciélagos son incapaces de mantener la replicación del DENV y es poco probable que actúen como reservorios de este virus.

Las enfermedades infecciosas continúan emergiendo y han sido reconocidas como una amenaza significativa para la salud humana, particularmente en países en desarrollo (Kuzmin *et al.* 2011). Durante las últimas décadas, el contacto entre los humanos y la vida silvestre ha sido asociado con la emergencia y re-emergencia de éstas enfermedades (Atlas *et al.* 2010). Sin embargo, se desconocen muchos aspectos sobre el papel que tiene la vida silvestre, aún en enfermedades tan conocidas e importantes para estos países como es el dengue. Hasta hace poco, los murciélagos raramente eran considerados como hospederos probables por los expertos que buscan virus emergentes. Actualmente se sabe que son los reservorios naturales de enfermedades emergentes como los virus Nipah y Hendra, y de otros patógenos más conocidos como el Virus de la Rabia (Dobson 2005). Más de 200 virus de distintas familias incluyendo a la que pertenece el DENV (Flaviviridae) han sido aislados o detectados de diferentes especies de murciélagos (Chu *et al.* 2009, Moratelli and Calisher 2015). Sin embargo, esto *per se*

no indica que papel tienen los murciélagos en la transmisión de estos virus, si son huéspedes incidentales o posibles reservorios.

Los murciélagos representan un casi el 25% de todas las especies de mamíferos y esta alta diversidad de especies y su amplia distribución mundial, contribuye a la gran gama de diferentes patógenos que pueden infectarlos (Bennett 2006). Asimismo, su gran longevidad relativa asociada a la posibilidad de que pueden presentar infecciones persistentes con ciertos virus, permiten mantener los virus por largos periodos de tiempo lo que aumenta la posibilidad de transmitirlos a otros vertebrados. Igualmente, su comportamiento gregario puede potenciar altamente la transmisión de los virus intra y entre especies (Dobson 2005, Calisher *et al.* 2006). Poseen una gran cantidad de especializaciones tróficas, siendo la mayoría especies frugívoras o insectívoras, y en Centro y Sur América, hay tres especies hematófagas. La disminución de recursos ambientales y la expansión urbanística en los hábitats naturales de los murciélagos pueden disminuir las fuentes de alimento natural. Esta habilidad de utilizar una amplia variedad de fuentes alimenticias puede llevar a un aumento en la biodiversidad en una pequeña área, potenciando la oportunidad de múltiples especies de interactuar y compartir agentes infecciosos (Kuzmin *et al.* 2011). Conjuntamente, los factores ambientales como períodos de escasez de recursos, pueden moldear la transmisión de patógenos a nuevas especies. Por ejemplo, durante una temporada seca, los monos y los murciélagos pueden estar en estrecho contacto, al presentarse un limitado suministro de recursos alimenticios, potenciando la posibilidad de transmisión entre especies (Kuzmin *et al.* 2011).

Además, contrario a cualquier mamífero, los murciélagos tienen la capacidad de volar, y esa extensa movilidad, asociada a su plasticidad en sitios de refugio y su amplio rango de alimentación, favorece que los murciélagos puedan transportar material viral a otros lugares y que éste entre en contacto con distintas especies de animales (Calisher *et al.* 2006). Por otro lado, los murciélagos presentan una amplia variedad de nichos ecológicos y su habilidad de vivir en estructuras hechas por el ser humano es de gran importancia, ya que aumenta la probabilidad de interacción entre murciélagos, animales domésticos y humanos (Bennett 2006). Por ejemplo, en los trópicos, los murciélagos

frugívoros se pueden encontrar en refugios urbanísticos, alimentándose de plantaciones de árboles frutales (Kuzmin *et al.* 2011).

Sin restarle importancia a la investigación con murciélagos como posibles reservorios virales, todo esfuerzo debe tomarse para evitar un abordaje reduccionista a las enfermedades infecciosas de manera que los murciélagos no se conviertan simplemente en objeto de persecución y erradicación (Wibbelt *et al.* 2010). Es responsabilidad de la comunidad científica, comunicar los hechos científicos y proveer un mejor entendimiento de los posibles riesgos, pero además, fomentar una coexistencia beneficiosa entre murciélagos y humanos y recordar que los murciélagos son de fundamental importancia para el ecosistema global, aún si portan enfermedades potencialmente perjudiciales para el ser humano (Wibbelt *et al.* 2010).

La ubicación privilegiada de Costa Rica en el istmo favorece a la gran biodiversidad de murciélagos, representando el orden de mamíferos más grande (LaVal & Rodríguez-Herrera 2002). Según un estudio de Drews (2002) realizado en Costa Rica, se determinó que 10 de cada 100 casas se encontraban colonizadas por murciélagos y los habitantes de las comunidades reportaban la presencia de murciélagos en edificios de la vecindad (escuelas, iglesias). Se observó que la convivencia con murciélagos es bastante común y se da en mayor grado en sitios urbanos. Aunque los murciélagos brindan grandes beneficios a los seres humanos, en términos de control de plagas, regeneración de bosques y polinización de cultivos (Melo *et al.* 2009, Boyles *et al.* 2011), entre otras, la percepción que las personas tienen de ellos es bastante negativa, inclusive en temas como la transmisión de enfermedades (Drews 2002). Sin embargo, no se sabe qué tipo de papel puede tener este mamífero en una enfermedad de tanta importancia como es el dengue. ¿Los afecta la infección, serán hospederos accidentales, replicarán el virus o servirán de reservorios?

El presente estudio, busca dilucidar si el murciélago es un posible reservorio de DENV, y si tiene un rol en el ciclo de transmisión viral, en el lugar donde podrían interactuar el humano, mosquito y murciélago: los sitios domiciliares. Además, de lo novedoso de la investigación, esta aproximación tiene enfoques desde distintas

perspectivas del problema: diagnóstico molecular y serológico, relación filogenética del agente viral que se encuentra en humanos, mosquitos y murciélagos; y la integración con factores antropogénicos y ecológicos que podrían moldear el ciclo de transmisión. Todos estos enfoques podrían aportar conocimiento en la descripción de un posible novedoso ciclo murciélago-mosquito-ser humano.

Capítulo 1. Dengue virus ecology in household environment: are bats reservoirs, hosts or accidentally involved in virus transmission?

Abstract

Dengue is the most important vector borne human disease worldwide. The importance of wildlife as reservoirs or hosts in (re)emerging infectious diseases has been constantly reported. Recently, some studies have shown Dengue Virus (DENV) nucleic acids and/or antibodies present in neotropical wildlife including bats. Thus it is possible that some bat species may be susceptible to DENV infection. This research aims to elucidate the role of house-roosting bats in a dengue virus transmission cycle. Bats were sampled in high and low dengue incidence regions during rainy and dry seasons in Costa Rica, where dengue is considered a hyper endemic disease with co circulation of all four serotypes. We captured 318 bats from 12 different species using mist nets from 29 houses where humans and bats cohabit. Permission for bat handling and euthanasia was obtained from the Institutional Committee of Care and Use of Animals of the University of Costa Rica according to international animal welfare standards. Necropsies were performed in 205 bats to analyze possible virus replication in heart, lung, spleen, liver, kidney, and brain tissue. We did not found the presence of DENV RNA in any organ. Blood samples were collected from all specimens and analyzed by neutralization and PCR. We obtained a seroprevalence of 22% (54/241). DENV RNA was detected in 8.8% of the bats sampled (28/318). From these 28 bats, we analyzed 11 intestine samples by PCR and two individuals were DENV RNA positive, corresponding with the same detected dengue serotype in blood. Viral isolation was attempted without any successful results. Additionally viral load analysis by qRT-PCR showed a very low virus concentration in positive blood samples, suggesting a virus concentration under the minimal threshold needed for mosquito infection by feeding. Simultaneously, 651 mosquitoes were collected using EVS-CO_2 traps and analyzed for the detection of DENV and bat cytochrome b. Only three were found positive for dengue and all of them were negative for bat cytochrome b. Our results suggest an accidental presence of dengue virus nucleic acids in bats and that their infection results possibly from oral ingestion of infected mosquitoes. Phylogenetic analyses suggest even a spillover event from human dengue outbreaks to bats. Therefore we conclude that bats do not seem to sustain DENV amplification and do not have a role as reservoirs, but represent an epidemiological dead end for this virus.

Keywords: dengue, house-roosting bats, spillover, dead-end host, accidental host.

INTRODUCTION

Dengue Virus (DENV) is the etiologic agent of dengue fever and its more severe forms, dengue hemorrhagic fever (DHF)/ dengue shock syndrome (DSS). DENV has a positive single-strand RNA genome, and belong to the genus Flavivirus (Guzman *et al.* 2010, Murrell *et al.* 2011). Dengue is the most important arthropod-borne viral infection of humans so far, it has been established in the tropics worldwide, and its geographical expansion is expected to increase due to factors such as the modern dynamics of climate change, globalization, travel, trade, poverty, unplanned urbanization, and also viral evolution (Murray *et al.* 2013). Dengue transmission occurs throughout the year in endemic tropical areas, however, a distinct cyclical pattern associated with the rainy season and the increase of mosquito abundance also exists (Feng and Velasco-Hernández 1997). The World Health Organization (WHO) estimates that 2.5 billion people are at risk of infection, with 50–100 million infections per year (OMS and TDR 2009). However, a study using cartographic approaches, estimated even higher numbers: 390 million dengue infections per year, of which 96 million will be clinically apparent (any level of disease severity) (Bhatt *et al.* 2013).

There are four distinct DENV serotypes (DENV-1–4), and infection with any of the four viruses results in lifelong immunity to that specific serotype (Murray *et al.* 2013). The ancestor of these viruses has been postulated to have emerged about 1 000 years ago in an infectious cycle involving non-human primates and mosquitoes, with transmission to humans occurring independently for all four virus types only a few hundred years ago (Wang *et al.* 2000, Rico-Hesse 2003, Twiddy *et al.* 2003, Vasilakis and Weaver 2008, Vasilakis *et al.* 2008, 2011, Weaver and Vasilakis 2009, Hanley *et al.* 2013). Currently, two distinct and independent DENV transmission cycles occur: (1) Endemic DENV circulates among humans, which serve as both reservoir and amplification hosts, and peridomestic *Ae. aegypti* and *Ae. albopictus*, with other *Aedes* spp. serving as secondary vectors; and (2) Sylvatic DENV circulates among non-human primate reservoir hosts and several different *Aedes* mosquitoes in forested habitats of

West Africa and Southeast Asia (Weaver and Vasilakis 2009). In the New World, unlike the Old World, it is believed that dengue infection is absent in wildlife as the introduced viral transmission cycle was the endemic cycle, with the appearance of its vectors *Ae. aegypti* and *Ae. albopictus* in the Neotropics (Weaver and Vasilakis 2009). However, several studies have documented the presence of DENV RNA and/or antibodies against DENV in mammals wildlife in the Neotropics (Platt *et al.* 2000, de Thoisy *et al.* 2004, 2009, Aguilar-Setién *et al.* 2008, Lavergne *et al.* 2009, Machain-Williams *et al.* 2013, Sotomayor-Bonilla *et al.* 2014). Nonetheless, it is unknown which mosquito species might function as transmission vectors in the forest, since *Ae. aegypti* is usually associated with urban sites (Lavergne *et al.* 2009).

Platt *et al.* (2000) conducted a study in Costa Rica and Ecuador where antibodies against DENV in bats were found by plaque reduction neutralization test (PRNT). Also, they observed that *Ae. aegypti* fed on bats in controlled laboratory conditions. Therefore, they suggested that these mammals could play a role in the cycle of the virus as possible reservoirs. In French Guiana, another study examined the presence of antibodies against flavivirus by serum neutralization tests in species of wild mammals of pristine forests (de Thoisy *et al.* 2004). They found neutralizing antibodies against DENV-2 in armadillos, porcupines, opossums, agoutis and wild goats. The authors proposed that wild animals can be exposed to DENV; and although their role in the transmission cycle is not known, they may function as temporary reservoirs with transmission by *Ae. aegypti* or other possible vectors. A study conducted in endemic areas of high dengue incidence in the Pacific coast of Mexico found by ELISA (enzyme-linked immunosorbent assay), RT-PCR (reverse transcription-polymerase chain reaction) and the presence of viral protein DENV NS1 (produced during acute infection and correlated with viral replication), four species of bats positive for DENV-2 (Aguilar-Setién *et al.* 2008). The authors suggested that although they do not rule out that bats could amplify the virus and act as reservoirs, that it is possible that they may play a role in preserving the DENV in nature. Another study in French Guiana, detected all four serotypes in liver samples and/or serum of 92 wild mammals (Lavergne *et al.* 2009, de Thoisy *et al.* 2009).

By short sequence analysis of the C/prM region they determined that DENV-1, DENV-3 and DENV-4 were different from those co-circulating in humans at that time point in the same geographical area. Furthermore, the analysis for DENV-2 short sequences found in mammals showed that some wild strains seemed to diverge from concurrent human strains, though others were identical. This suggests that neotropical mammals living in peri-urban areas may encounter with DENV strains circulating in humans, and under pressure from a strong epidemic event, urban strains could be introduced into the forest and infect part of the wildlife fauna (Lavergne *et al.* 2009, de Thoisy *et al.* 2009). The authors emphasize that it is important to consider wildlife not only as potential reservoirs for DENV, but also as potential hosts, sensitive to infections by human pathogens (de Thoisy *et al.* 2009). They suggest that these species of mammals can be an epidemic dead end or play a role in maintaining the virus in epidemic interperiods. Another serological survey conducted in Mexico detecting antibodies against West Nile virus (WNV), St. Louis encephalitis virus (SLEV), and DENV in bats found flavivirus-specific antibodies in 19% of the bats (Machain-Williams *et al.* 2013). Here the PRNT titers against DENV-2 or DENV-4 were greater than for other flaviviruses, however all around, the neutralization titers were considered low. Therefore they conclude that it is possible that bats had been infected with another flavivirus not included in the analysis (Machain-Williams *et al.* 2013). Additionally, DENV was searched in bats captured from anthropogenically changed and unaltered landscapes in southern Mexico (Sotomayor-Bonilla *et al.* 2014). They found DENV-2 in the spleen of six bats and no effect of anthropogenic disturbance on the occurrence of DENV.

Two recent studies have tested the susceptibility of bats for DENV infection in laboratory conditions. Perea-Martínez *et al.* (2013) showed that after intraperitoneally inoculation of DENV-2, the virus replicates poorly on bats, suggesting that they are not suitable hosts for this virus. In addition, Cabrera-Romo *et al.* (2014) inoculated bats with DENV-1 and DENV-4 using different routes: subcutaneously, intraperitoneally, and bitten by infected *Ae. aegypti*. They could detect DENV RNA (6/22) in spleen and NS1 (8/22) in serum in some cases, but in low concentrations and they were not able to

reproduce their results. The authors conclude that bats are incapable of sustaining dengue virus replication and are unlikely to act as reservoirs for this virus.

More than 200 viruses from 27 different families including Flaviviridae have been isolated or detected in bats (Moratelli and Calisher 2015). However, whether bats are simply incidental virus hosts or serve as competent reservoirs able to transmit these viruses to other vertebrates are open questions that must be carefully addressed (Dobson 2005, Calisher *et al.* 2006, 2008, Bennett 2006, Wong *et al.* 2007, Wibbelt *et al.* 2010, Melaun *et al.* 2014, Moratelli and Calisher 2015). Bats are extremely important components of biodiversity: their role in forest regeneration and insect pest control is well known (Melo *et al.* 2009, Boyles *et al.* 2011). The privileged geographical location of Costa Rica favors the rich bat biodiversity, representing this land largest order of mammals (LaVal and Rodríguez-Herrera 2002). In Costa Rica, 10 out of 100 houses are colonized by bats, and community reports of bats colonization in neighbouring buildings such as schools and churches are common (Drews 2002). This indicates a close proximity with these wild animals in this country.

The present study aims to have a comprehensive view of a putative cycle of dengue viral transmission involving humans, mosquitoes and bats in places where they could interact: household environments. In addition, this research has approaches from different perspectives of the problem: molecular and serological diagnosis, phylogenetic relationship of the viral agent and integration with anthropogenic and ecological factors that could shape a possible transmission cycle.

METHODS

Sampling

Sampling was performed in the rainy and dry season between 2013 and 2014 in three different locations of dengue low and high incidence, according to the infection of

the dengue vector mosquitoes and the reported cases of dengue given by the Ministry of Health (2015) (Fig.1). The first site, La Virgen from Sarapiquí, is a rural area that belongs to the costarrican Caribbean slope. This site is surrounded by rainforest, and agricultural fields of mainly pineapple and banana, and presents high incidence of dengue. The second site, Nicoya, is a peri-urban area from the Pacific coast, located between pastures and dry forest, with dengue high incidence. The third site was the Central Valley, located in the middle of the country where the Great Metropolitan Area is found. This site has low incidence of dengue. At each site, houses on which humans and bats cohabit where located, and sampled on dry and rainy season. We attempted to sample the same house if bats were still present after the first sampling. If not, a nearby house was searched and sampled.

After identifying the houses, bats were captured with mist nets positioned at their root's exit or directly per hand from the ceiling, with leather gloves. All specimens were collected following the recommendations of the Institutional Committee of Care and Use of Animals of the University of Costa Rica (CICUA-36-13) according to international animal welfare standards. Captured animals were taxonomically classified using external morphological characteristics (Timm *et al.* 1999). Age, sex, and reproductive status were also determined. Five bats per household were euthanized by cardiac puncture exsanguination under anesthesia (ketamine 10 mg/kg + xylazine 1 mg/kg). Necropsies were performed collecting aseptically heart, lung, liver, spleen, kidney, and brain. A fraction of each organ was preserved at -80°C with 200 µl of ARNlater® Stabilization Solution (Life Technologies, Thermo Fisher Scientific Inc.). Another fraction was preserved in 10% neutral buffered formalin for histopathology analyses. Left material from carcasses was deposited at the Zoology Museum of the University of Costa Rica for other studies. The remaining bats captured from each household were released after blood sampling by puncture of a branchial vein. Blood collected from all specimens was centrifuged for 10 min at 3500 rpm to obtain plasma. Coagula and plasma were store at -80°C for later analysis.

On the sampling day, four EVS-CO_2 Traps (BioQuip Products, CA, USA) were placed inside and outside of each house. They were placed before dusk until the middle of the morning on the next day. Collected mosquitoes were frozen in dry ice, identified to species or genus (Chaverri 2009), and stored at -80°C for later PCR analysis. Mosquito breeding sites near or inside the households were also located. Larvae were collected, and under laboratory safety left to become adults for identification. This broad mosquito sampling was done in order to include all possible mosquito species present at the site of study, and not only sampling *Aedes* mosquitoes, since we did not know if there could be any other species participating in a possible sylvatic cycle.

A survey was conducted in each household to determine awareness of previous dengue infections, social and economic aspects, and their interaction with bats. After signing an informed consent approved by the university ethics committee according to the principles expressed in the Declaration of Helsinki (CEC VI-3970-2013), a blood sample was taken. Blood collected was centrifuged for plasma separation for storage at -20°C for further ELISA analysis.

Bats

Molecular methods:

Viral RNA was extracted from blood and from a pool of collected organs using TRIzol Reagent® (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 10 µl of total RNA using RevertAid™ H Minus Kit (Fermentas®, ThermoFisher Scientific, USA) with random hexamers or the D1 forward primer (Lanciotti *et al.* 1992), according to the manufacturer's instructions. A seminested-PCR was performed using the TopTaq Master Mix (Qiagen, Germany) and the primers following Lanciotti *et al.* (1992). Briefly, PCR was performed using 5 µl of cDNA and the D1 and D2 primers, amplifying a fragment of 511bp corresponding to a portion of the capsid and premembrane (C/prM) genes. The PCR program consisted of an initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation (94°C, 30s), annealing (55°C, 30s), and extension (72°C, 30s), with a final elongation for 10

min at 72°C. The second amplification was performed using 2 µl of an 1:100 dilution of the first PCR product, using the Multiplex PCR kit (Qiagen, Germany) and the primers D1 and TS1-TS2-TS3-TS4, generating PCR products of different sizes for each serotype (482bp for DENV-1, 119bp for DENV-2, 290bp for DENV-3, and 392bp for DENV-4). The amplification consisted of an initial denaturation at 95°C for 5 min, then 30 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min, followed by a final incubation at 72°C for 10 min. All the positive and negative controls were present in each run for serotype confirmation and to rule out cross-contamination. The products were visualized on a 1.5% agarose gel containing GelRed™ (Biotium, Hayward, CA) under UV light with the ChemiDocTM XRS+ System (BIO-RAD). When a bat was positive in whole blood for viral RNA, single organ (heart, lung, liver, spleen, kidney, brain and intestine (when collected) PCR analyses was performed.

Positive samples for DENV RNA were further analyzed by a quantitative Real Time PCR (qRT-PCR) assay, as described by Drosten *et al.* (2002). A StepOne™ RT-PCR System (Applied Biosystems, ThermoFisher Scientific) with the SuperScript III OneStep RT-PCR System with Platinum Taq kit (Invitrogen, ThermoFisher Scientific) and the primers and probe (DEN IVT) designed by Drosten *et al.* 2002 were used. A total of 25 µl reaction mixture, including 5 µl template RNA, were run under the cycling conditions: one RT step at 45°C for 30 min, a denaturation step at 95°C for 5 min, 45 cycles of 95°C for 5 s and 57°C for 35 s single read step F1/F2. Quantification was performed using a standard curve generated with dilutions of the probe

Virus isolation:

Additionally viral isolation was attempted from PCR-DENV positive blood samples. 48-well flat-bottomed cell culture plates (Cellstar®, Greiner Bio-One, Germany) were seeded with 2.5 x 10E5 C6/36 (*Aedes albopictus* cell line) in 500 µl in RPMI 1640 medium with GlutaMAX™-I (Gibco®, BRL) supplemented with 2% fetal bovine serum (Gibco®, BRL), penicillin (100 units/ml) and streptomycin (100ug/ml) (Sigma-Aldrich, USA). Coagula with plasma rest were washed with 25-50 µl of sterile PBS, centrifuged and 20 µl of the supernatant were inoculated to each of two wells per

sample. Cells were incubated at 28°C in a 5% CO_2 atmosphere during 24 hours for virus adsorption, then medium was changed and further incubated during 15 days, with a medium change every week. Afterwards, cells were passage into a 25cm^2 cell culture flask (Cellstar®, Greiner Bio-One, Germany), and incubated at 28°C 5% CO_2 for 30 more days, with a weekly change of medium. Cells were observed daily for appearance of viral cytopathic effect (CPE), and analyzed for DENV ARN by PCR.

Microneutralization assay:

Bat serum was analyzed in a microneutralization assay performed in 96-well, flat-bottomed tissue culture plates (Corning, NY, USA). Plates with Vero cells were seeded at 3 x 10E4 cell/well with minimal essential medium (MEM), containing 10% fetal bovine serum (FBS) (Gibco®, BRL) and incubated for 24 h at 37 °C 5% CO_2 until monolayer was confluent. Serum samples were heat-inactivated at 57 °C for 30 min, and diluted 1:10 in MEM 2 %. 30 µl of the virus inoculum with different PFU amounts (DENV-1 Angola (D1/AO/XX/1988), 100 PFU; DENV-2 Jamaica (D2/JM/1409/1983), 150 PFU; DENV-3 Nicaragua (D3/NI/30-94/1994), 150 PFU; DENV-4 Dominica (D4/DM/ 814669/1981), 200 PFU) were mixed with an equal volume of serum dilution (final dilution 1:20) and incubated 1h at 37°C. Then, 50 µl of the serum-virus mixture was placed into Vero cultures and incubated 90 min at 37°C. After adsorption, the serum-virus inoculum was removed and 100 µl of 1.5% carboxymethylcellulose (CMC) (Sigma-Aldrich, USA) overlay medium consisting of cell culture medium supplemented with 5% heat inactivated FBS (Gibco®, BRL), 3% sodium bicarbonate (Sigma-Aldrich, USA), 1% antibiotic/antimycotic solution (Sigma-Aldrich, USA) were added. Plates were incubated at 37°C in an atmosphere of 5% CO_2 for 72 hours for DENV-1, DENV-2, and DENV-3, and for 48 hours for DENV-4. As assay controls, a positive human serum pool (sera previously determined to possess high ELISA titers against all four DENV serotypes), negative human serum, and mock cell controls were included. After the incubation period, the CMC overlay medium was removed. After washing twice with PBS, cells were fixed with absolute methanol at -20°C for 12 h. For detection of virus infected cells, immunostaining of plaques was performed. Plates were washed once

with PBS and incubated 30 min with a 1:200 dilution of monoclonal Dengue virus 1, 2, 3 & 4 antibody [D1-11(3)] (GeneTex, CA, USA) for DENV-1, DEN2 and DENV-3; and with an 1:200 dilution of mouse anti-WNV IgG 4G2 antibody (Hennessey Research, Inc.) for DENV-4. After washing, a 1:200 dilution of Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) was used for detection. All antibodies were diluted in 0.1% nonfat milk in PBS as a blocking solution. Viral plaques were visualized after 30 min incubation with a freshly prepared solution of 3-amino-9-ethyl carbazole (AEC) (Sigma-Aldrich, USA) (1 mg/mL in 50mM acetate buffer, pH 5-5.5) containing 0.3% H_2O_2. Washing steps were implemented in between each antibody and substrate incubation. The virus-infected foci were counted to determine 90% of virus neutralization.

Mosquitoes

Mosquitoes were sorted by date, house, trap and species. Only female mosquitoes were dissected in heads and abdomens. Forceps and surgical blades used were sterilized in ethanol, flamed and immersed in DNA Away (Molecular Bioproducts Inc., CA, USA) between dissections to avoid cross-contamination. Pools of 25 or fewer individuals were macerated and homogenized in 200 µl of RNAlater® Stabilization Solution (Life Technologies, Thermo Fisher Scientific Inc.). Viral RNA extraction, cDNA retrotranscription and DENV PCR were performed as described above.

For blood meal analysis of mosquitoes' guts, DNA was extracted from pools using NucleoSpin® Tissue (Macherey-Nagel, Germany) according to the manufacturer's instructions. Gene segments were amplified from 5 µl of DNA using TopTaq Master Mix (Qiagen, Germany) and two sets of primers that amplify overlapping regions of mitochondrial cytochrome oxidase subunit I (COI), COI_short and COI_long, and one primer set for cytochrome b (Cyt *b*) as described in Townzen *et al.* (2008). The amplification protocol for both genes was: 95 °C for 1 min; 35 cycles of 95 °C for 30 s, 50°C for 50 s, 72 °C for 1 min; and a final extension cycle at 72 °C for 5 min. Negative controls were run with each extraction and PCR reaction. PCR products were verified by

1.5% agarose gel electrophoresis containing GelRed™ (Biotium, Hayward, CA). PCR products were purified using Exonuclease I and Thermo Scientific FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific Inc.) following the manufacturer's protocol. Both strands of the amplicons were sequenced by Macrogen Inc. (Seoul, South Korea) using the amplification primers. To confirm identity of the vertebrate blood present in the gut of the mosquito pools, each sequence was compared with entries of homologous sequences contained in GenBank using the nucleotide – nucleotide basic alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/).

Humans

DENV-specific IgG titers in human serum samples were determined by a sandwich-like enzyme-linked immunosorbent assay (ELISA) system (AccuDiag TM Dengue IgG ELISA kit; Diagnostic Automation, Inc., Calabasas, CA). Analysis of the samples and the interpretation of positive or negative ELISA reactions were made according to the manufacturer's instructions.

Phylogenetic Analysis

After dengue serotype identification of positive samples from bats and mosquitoes, we amplified cDNA segments by two different methodologies for phylogenetic analysis. First, cDNA segments between 2,474 and 2,577 nucleotides encompassing the prM and E genes of DENV were amplified by PCR using the consensus primer D1 and serotype-specific reverse primers as in Díaz *et al.* (2006a). 10 µl of the cDNA and the TopTaq Master Mix (Qiagen, Germany) were used, following the manufacturer protocol. For DENV-1, DENV-2 and DENV-4, the PCR program consisted of an initial denaturation at 95°C for 5 min, followed by 10 cycles of denaturation (95°C, 45s), annealing (54°C, 2 min), and extension (68°C, 2 min), then by 25 cycles of denaturation (95°C, 45s), annealing (65°C, 2 min), and extension (68°C, 3 min), with a final elongation for 15 min at 72°C. For DENV-3, the annealing temperature was changed to 51°C and 60°C, respectively. Additionally, cDNA segments

between 424bp and 461bp including the C/prM region or DENV were amplified using the first PCR products from the Lanciotti *et al.* (1992) seminested-PCR, for another seminested PCR amplification using D1 and one of serotype-specific reverse primers, as described in de Thoisy *et al.* (2009). The amplification protocol was: an initial denaturation at 94°C for 5 min, then 25 cycles at 94°C for 30 s, 52°C for 90 s, and 72°C for 60 s, followed by a final incubation at 72°C for 10 min. PCR products were verified by 1.5% agarose gel electrophoresis. PCR products were purified using Exonuclease I and Thermo Scientific FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific Inc.) following the manufacturer's protocol. Both strands of the amplicons were sequenced by Macrogen Inc. (Seoul, South Korea), using either the primers from Díaz *et al.* (2006) or the amplification primers from De Thoisy *et al.* (2009).

Obtained sequences from bats, mosquitoes and from already published dengue isolates from the 2007 outbreak in Costa Rica (Soto-Garita *et al.* 2016) were aligned with previously published sequences of dengue virus submitted to GenBank (Supplementary Table 2 and Supplementary Table 3) using MEGA v6.0 software, and alignments were checked manually. The identification of the best nucleotide substitution model and the construction of phylogenetic trees were performed using MEGA v6.0 (www.megasoftware.com). The robustness of the resulting tree was established by bootstrap analysis with 1 000 replications.

Statistics

For the DENV RNA results, factors such as gender, site (Sarapiquí, Nicoya or Central Valley), and season (dry or rainy) were subjected to the chi-squared test (χ^2). While for the seropositivity, the factors such as gender, age (juvenile or adult), reproductive status (inactive, pregnancy, lactancy), and site were tested. A GLMM Binomial was performed in order to test correlation between human IgG anti DENV presence and bat DENV RNA presence. Mosquito abundance was tested by site and season with Kruskal-Wallis. Analyses of the data were done using R v3.2.1 software.

RESULTS

Bats

A total of 318 bats from twelve species were captured in 29 different houses. From this number, 205 were euthanized and a blood sample was taken from 113. After an initial screening for DENV RNA in blood, 60 possible positive bats were obtained. For confirmation, the PCR was repeated and the DENV RNA amplicon from the semi-nested PCR was sequenced, and compared with entries in the database of homologous sequences contained in GenBank using BLAST. Dengue viral RNA from the four serotypes was detected and confirmed in the blood of 8.8% of the bats samples (28/318; Table 1). DENV-1 was present in 17.8% (5/28), DENV-2 in 50% (14/28), DENV-3 in 7% (2/28), and DENV-4 in 35.7% (10/28). Interestingly, we found two individuals presenting double infections with DENV-2 and DENV-4, and one with DENV-2 and DENV-3.

Geographically, at Sarapiquí 7.8% of the bats sampled (8/102) were infected with DENV-2 (3/8), DENV-3 (2/8), and DEN4 (3/8). At Nicoya, 6.8% of the bats sampled (6/88) were infected with DENV-2 (3/6) and DENV-4 (3/6). Finally, at the Central Valley, 14.4 % of the bats sampled (17/118) were infected with DENV-1 (5/17), DENV-2 (8/17) and DENV-4 (4/17). Surprisingly, the Central Valley was the only site where DENV RNA was detected during dry season (7 positive bats) even though we considered it a low dengue incidence region. We found no differences of DENV infection, between gender (χ^2= 0.09, df=1, p=0.76), site (χ^2= 3.40, df=2, p=0.18) or season (χ^2= 1.87, df=1, p=0.17).

To detect putative viral replication sites in bats organs, we analyzed the organ pools for DENV RNA presence. We did not detect any DENV RNA in the pool of organs. To exclude possible dilution of the DENV RNA in the pool hence the negative results, we analyzed then each organ individually from the bats with DENV-positive blood. Interestingly, nor heart, lung, liver, spleen, kidney, or brain were found positive

for viral RNA. From the 28 DENV RNA positive bats, we had collected the intestine from eleven of them. Surprisingly, after DENV RNA analysis two individuals had positive DENV RNA PCR results, coinciding with the previously detected serotype in their blood.

We attempted virus isolation from DENV RNA positive blood samples in C6/36 cells. After 2 days some of the samples showed CPE, though with an atypical appearance for Dengue. RT-PCR analysis of all supernatants and cells resulted negative even 45 days after sample inoculation. To rule out the possibility that virus present in the blood was in low quantity and therefore precluded successful isolation, the quantitative RT-PCR was done. The qRT-PCR of all positive samples showed to be under the detection of the standard curve (16.4 copies/µl), which indicates probably a very low concentration of the virus in the blood samples.

For testing for the presence of anti-dengue antibodies, a microneutralization test was performed in 241 serum from the bats sampled. We found an all-around DENV seroprevalence of 22% (54/241; Table 2). There were no differences in the seroprevalence between males and females (χ^2= 0.35, df=1, p=0.55) nor juveniles and adults (χ^2= 0.03, df=1, p=0.86). Antibodies against DENV-1 were present in 24.5% (13/53) of the total positive bats, against DENV-2 in 22.6% (12/53), DENV-3 in 67.9% (36/53), and DENV-4 in 3.8% (2/53), with twelve individuals presenting antibodies against more than one serotype. Sarapiquí presented higher seroprevalence (χ^2= 7.31, df=2, p=0.03), with 30.67% of the bats sampled presenting antibodies against DENV (23/75), specifically against DENV-1 (5/23), DENV-2 (7/23), and DENV-3 (18/23). At Nicoya the seroprevalence was lower at 18.9% (14/74), with the presence of antibodies against all four serotypes, DENV-1 (5/14), DENV-2 (1/14), DENV-3 (9/14), and DENV-4 (2/14). Lastly, at the Central Valley DENV seroprevalence was slightly lower at 16.67% (14/84), presenting antibodies against DENV-1 (3/14), DENV-2 (4/14), and DENV-3 (9/14).

Six individuals presented antibodies and DENV RNA in blood at the same time. In three occasions the antibodies present were against the same serotype detected in blood, suggesting an immunological response. In the other three individuals, the antibodies present were not against the serotype found in blood, showing to be a potential second infection by another serotype.

Mosquitoes

To analyze if mosquitoes collected were dengue positive and had bitten bats, we set EVC CO2 traps in each household were bats were captured. We captured 651 mosquitoes, 121 males and 531 females, from the following species: *Culex quinquefasciatus* (202), *C. nigripalpus* (315), *C. mollis* (1), *C. lactator* (16), *Culex* sp. (29), *Aedes aegypti* (54), *Ae. albopictus* (4), *Aedes* sp. (21), *Trichoprosopon digitatum* (2), *Limatus durhamii* (1), *Anopheles apicimacula* (2), *An. neivai* (1), and *Anopheles* sp (3). We made 39 pools of males and 108 pools of females. The female pools were then subdivided in heads and bodies. The *Aedes* species mosquito was present in all sampling sites, though in Sarapiquí we only found *Ae. albopictus*. All the *Ae. aegypti* were found in Nicoya in the rainy season, with just two *Ae. aegypti* captured in the Central Valley. We found no difference of mosquito abundance between sites (Kruskal-Wallis, χ^2= 3.99, df=2, p=0.14). Although not statistical significant (Kruskal-Wallis, χ^2= 3.99, df=2, p=0.14), overall more mosquitoes were collected during rainy season, but in the Central Valley where we found more mosquitoes during the dry season. Surprisingly, in one household sampled in the Central Valley (which is considered to have low dengue incidence in humans), DENV-1 was detected in one *Ae. aegypti* (head and body pool) and DENV-2 in a *Culex* sp. (body pool). On the other hand DENV-3 was detected in a headpool of *Culex* sp. from Sarapiquí. All the body pools were tested for blood meal preference by detection of *Cyt b*. We found human, dog, cat, rooster, horse, cattle and rat blood, but no bat blood was found.

Humans

At the time of sampling, no person presented any dengue symptomatic infection. As expected, Nicoya presented higher seroprevalence in humans by ELISA with a positivity of 82.6% (19/23). In Sarapiquí the seroprevalence was 16.7% (4/24) and in the Central Valley was 8.3% (1/12). Our results go in agreement with the epidemiological data published by the Ministry of Health, where in 2013 the incidence rate was 6592.1 in Nicoya, 2236.6 in Sarapiquí, and 2023.6 in the Central Valley (in the districts were we sampled). Whereas in 2014, the incidence was 677.4 in Nicoya, 773.8 in Sarapiquí, and 134 in the Central Valley (Ministerio de Salud 2015; Fig. 1). However, we found no correlation between human anti dengue IgG prevalence and bat dengue positivity among the sampled houses (GLMM Binomial, $Z=-0.964$, $P>0.05$, $N=235$). Despite the fact that people indicated that they have not travelled outside their living town 15 days prior of sampling, we could not be certain that the infection took place in their own houses or somewhere else.

Phylogenetic Analysis

In order to compare if viruses detected in bats, mosquitoes and humans were similar, we sequenced our PCR products and did phylogenetic analysis. It was only possible to obtain good quality C/prM sequences from DENV-2:10 from bats and one from mosquito; and from DENV-4: 7 from bats. Phylogenetic trees were assembled using the maximum likelihood (ML) statistical method based on K2 + G for both DENV-2 and DENV-4. The phylogenetic tree retrieved from the C/prM sequence of DENV-2 is shown in Figure 2. A total of 49 DENV-2 sequences were used for this analysis: 25 from GenBank, 3 from a past outbreak and 11 new sequences from this study (Fig. 2; Supplementary Table 2). The retrieved sequences cluster together in the Asian/American genotype, the same genotype of strains from Nicaragua and Costa Rica. A total of 31 C/prM sequences of DENV-4 were included in the phylogenetic analysis: 24 from GenBank and 7 from bats (Fig. 3; Supplementary Table 3). The sequences of bat cluster together in Genotype II, along with other sequences of DENV 4 reported

from Costa Rica where a mosquito sequence was included (KJ534635.1, Calderón-Arguedas *et al.* 2015).

DISCUSSION

Flaviviruses are zoonoses that depend on animal species other than humans for their maintenance in nature, though dengue viruses are an exception (Gubler 2001). Dengue viruses have completely adapted to humans and are maintained in large urban areas in the tropics in human-mosquito transmission cycles that no longer depend on animal reservoirs, although such reservoirs are still maintained in the jungles of Africa and Southeast Asia in non-human primate-mosquito transmission cycles (Gubler 2001, 2002, Mackenzie *et al.* 2004). Recent studies have shown the presence of antibodies against DENV and/or DENV RNA in wildlife from the Neotropics, including bats (Platt *et al.* 2000, de Thoisy *et al.* 2004, 2009, Aguilar-Setién *et al.* 2008, Lavergne *et al.* 2009, Machain-Williams *et al.* 2013, Sotomayor-Bonilla *et al.* 2014). But it is still unclear whether bats can maintain the virus and serve as reservoirs. In the present study, the presence and exposure to DENV in house-roosting bats was determined and their possible role as reservoirs was evaluated.

Our results show that bats that cohabit with humans are critically exposed to DENV, since almost 9% sampled presented DENV RNA in blood and a 22% seroprevalence. However, our findings suggest that bats are accidentally infected by DENV since: i) Although some studies have found the presence of NS1 in blood, hence virus replication (Aguilar-Setién *et al.* 2008), we were not able to detect virus RNA in any putative replication organ (heart, lung, kidney, liver, spleen and brain); ii) Quantification of viral RNA by qRT-PCR in blood showed low RNA concentrations and these genome copies may not be enclosed in intact or infective viral particles. This low concentration is under the minimal mosquito infectious dose (MID) required to maintain the virus transmission cycle (Pongsiri *et al.* 2014). Moreover, the low concentration and

quality of virus RNA in blood precluded amplification of the 2.5 kbp region encompassing the prM/E genes (Díaz *et al.* 2006). Thus we obtained only small sequences (424-461bp) from the C/prM region with a seminested PCR for sequencing (de Thoisy *et al.* 2009). Finally, and iii) we failed virus isolation attempts from positive blood samples suggesting no intact or infectious virus present. Taken together, these results suggest that bats do not amplify the virus, excluding them as potential hosts or reservoirs, having no role in the transmission cycle, and making them feasible dead-end hosts for the virus. This confirms results obtained with previous independent studies where bats infected with DENV in controlled laboratory conditions failed to show viral amplification (Perea-Martínez *et al.* 2013, Cabrera-Romo *et al.* 2014).

DENV RNA detected from the 28 positive individuals corresponded to the four serotypes of dengue. Surprisingly 3 individuals presented double positive PCRs for different serotypes (2 with DENV-2 and DENV-3, and 1 with DENV-2 and DENV-4). The majority of positive samples were for DENV-2, followed by DENV-4, DENV-1 and to a lesser extent DENV-3. While serotypes 1, 2 and 3 are responsible for dengue outbreaks in this country during 2013-2015, DENV-4 has not been reported in human samples from Costa Rica since 2002 (Ministerio de Salud 2015). However DENV-4 was reported from an infected *Ae. albopictus* in a pineapple field in Sarapiquí (Calderón-Arguedas *et al.* 2015). Besides competing with the other three serotypes, it has been reported that DENV-4 is the least frequently isolated serotype, it has not been associated with severe dengue outbreaks, and causes most of the clinically mild cases after dengue infection (Klungthong *et al.* 2008, Kyle and Harris 2008). Thus, DENV-4 may circulate unnoticed without its detection in health care facilities or epidemiological surveillance services, and bats act as sentinels showing the circulation of this serotype.

We predicted to find more positive bats for DENV in the locations where dengue human incidence was higher. Yet we found dengue positive bats in all sites sampled independently from their reported human seroprevalence by the National Ministry of Health (Ministerio de Salud 2015). Also, we did not find any correlation between the seroprevalence against dengue in humans sampled during this research and the presence

of DENV RNA in bats. We also expected to find more positive individuals during the rainy season since the mosquito population augments and the cases of dengue in humans increase considerably (Feng and Velasco-Hernández 1997, Ministerio de Salud 2015). In Nicoya and Sarapiquí we did not find individuals positive for dengue during the dry season as expected, but in the Central Valley we found positive bats during both seasons. Despite the fact that we did not find a significant difference in the quantity of mosquitoes between seasons, overall we collected more mosquitoes during the rainy season than in the dry season. Interestingly in the Central Valley alone more mosquitoes were collected during the dry season. The presence of vector mosquitoes sustaining the viral transmission during the dry season has been associated with human habits such as saving water for the drought in artificial containers without proper management, therefore becoming potential breeding sites (Trewin *et al.* 2013). The hyper endemic circulation of dengue and the presence of the mosquito vector in all sampled households may indicate enough sources for bats infection. *Ae. aegypti* was present in almost all the households sampled during the rainy season in Nicoya. On the other hand, *Ae. albopictus* was the vector found in Sarapiquí, accordingly to previous studies where the expansion and increased population of *Ae. albopictus* due to changes in land use (i.e. recent pineapple fields) suggesting a possible competition with *Ae. aegypti* is reported (Calderón-Arguedas *et al.* 2012, 2015).

Furthermore, the high seroprevalence observed in bats suggests a high exposure and rate of contact between bats and DENV. We observed more antibodies against DENV-3 (67.9%) and most of them seemed to be from a relatively old infection (data not shown), perhaps from an older outbreak caused mainly by DENV-3. Considering that differences between age and gender have been detected in the immunological response against other viruses (Thompson *et al.* 2015), we tested if this was the case with bats and dengue. We found no significant difference, suggesting that males and females, as well as adults and juveniles, are equally exposed to DENV. However, we did find higher seroprevalence in Sarapiquí compared to the other two sites, although we expected to find more seropositivity in Nicoya as reported in humans (Ministerio de

Salud 2015). Sarapiquí has a greater amount of rainfall yearly with a less drastic dry season, nourishing populations of mosquitoes all year round, which may sustain viral transmission throughout the entire year. In six individuals we found concomitantly DENV RNA in blood and antibodies against DENV. Half of the individuals presented antibodies against the same serotype detected in blood, suggesting a previous or parallel to infection immunological response. The other three individuals presented antibodies against a distinct serotype as the detected in blood by RT-PCR suggesting a potential secondary infection. Studies indicate that even after a controlled infection with a virus, bats do not always present antibodies against this particular virus (Davis *et al.* 2005, Van Den Hurk *et al.* 2009). This observation displays how complex and different the immunological response in bats is, thus making serological results harder to interpret. Also, we have to take into account that more than 200 viruses have been described in bats, with representatives of 27 distinct virus families, including Flaviviridae (Moratelli and Calisher 2015). So far, at least 19 flaviviruses have been associated with bats (Charlier *et al.* 2002, Lamballerie *et al.* 2002, Davis *et al.* 2005, Tajima *et al.* 2005, Kuno and Chang 2006, Cui *et al.* 2008, Van Den Hurk *et al.* 2009, Wang *et al.* 2009, Epstein *et al.* 2010, Volkova *et al.* 2012, Quan *et al.* 2013, Machain-Williams *et al.* 2013, Cadar *et al.* 2014, Moratelli and Calisher 2015, Thompson *et al.* 2015), some of them corresponding to the group of unknown transmitting vector, while others are known to be transmitted by mosquitoes. Therefore, although PRNT is the gold standard for the serological diagnosis of flaviviral infections and can be highly specific, interpretation of the PRNT results from bats must be cautious since simultaneous assessment against endemic flaviviruses to a given area must be performed to allow comparison of end-point titers (Hobson-Peters 2012).

We expected that dengue bat infection was through an infected mosquito bite, but no mosquito was found positive for bat cytochrome b. However, our findings surmise that infection may occur through the ingestion of an infected mosquito. Previous studies mention the detection of antibodies and RNA in bats after the ingestion of mosquitoes infected with other flaviviruses such as Yellow Fever Virus (YFV) and WNV (Scott

2001, Davis *et al.* 2005, Melaun *et al.* 2014). Additionally, albeit we could not detect the virus in any putative replication organ, we detected in two intestines from *M. sinaloae* the same serotype of DENV as found in blood. Although this result may be caused by the presence of a positive dengue mosquito inside the lumen of the intestine, it is tempting to speculate that some limited local viral replication in the endothelial cells of the bat intestine may be occurring. Supporting this conjecture, failure in recent studies attempting infection of bats with dengue (with serotypes 1, 2 and 4) via the bite of an infected mosquito or the subcutaneously and intraperitoneally inoculation of the virus resulting in little or none viral replication and limited antibody response may indicate an oral infection route (Perea-Martínez *et al.* 2013, Cabrera-Romo *et al.* 2014). Accordingly the collected mosquitoes feeding preference did not indicate feeding on bats, even though the EVC-CO_2 traps were in close proximity to the roosting place from the bats. Moreover, the majority of the bats sampled are insectivorous, and even though the preferential food source for the Molossids and other bigger bats may not be insects as mosquitoes, it is possible that they will feed on them due to increased abundance in rainy season (Boyles *et al.* 2011, Gonsalves *et al.* 2013). Likewise, a nectarivorous bat as *G. soricina* could accidentally feed on dengue positive male mosquitoes while functioning as pollinators from flowers. Although the *Aedes* mosquitoes are from diurnal habits, other nocturnal mosquitoes as *Culex* sp. that have a negligible role in DENV transmission, showed limited virus replication in the gut (Vazeille-Falcoz *et al.* 1999). Bats could also feed on these mosquitoes that may carry enough viruses to produce an infection.

Even though bats seem to get infected with DENV, they do not seem to amplify the virus to a considerable extent to be able to transmit it to a mosquito. It seems that the exposure of bats to DENV is accidental, becoming an example of spill over from humans to bats as reported by de Thoisy *et al.* (2009) with samples of wildlife taken in close proximity to human settlements where dengue outbreaks occur. This is supported by our results from our phylogenetic analysis (Fig. 2 and Fig. 3), where the dengue strains sequenced from bats and mosquitoes cluster together with close relation with the

reported strains of dengue in this and neighboring countries. Also, bats no dot show any signs of inflammation or infection caused by the virus via histopathological analyses of all organs collected (data not shown). Although we cannot determine with certainty which is the route of contact between the virus and the bat, our results suggest that the bat is an epidemic dead end for the virus.

Several viruses have been isolated or detected in bat tissues or excreta; however, this does not prove a relationship between the presence of a virus and the disease the virus might cause (Calisher *et al.* 2008). Some of these viruses or viral sequences might have been in food eaten by bats and could be irrelevant with respect to viral disease epidemiology. Because of the many gaps in our knowledge linking bats and zoonotic viruses, associating bats with these events without any further evidence is a disservice, with negative consequences for bats and humans (Moratelli and Calisher 2015). Negative consequences for bats as they are considered pests that need control and fear dissemination among the general public. For humans, since investing efforts in controlling the wrong reservoir or disease carrier can postpone appropriate mitigation actions that could prevent more deaths or interrupt the spread of the disease. Also, a potential 'pest control' of bat populations may deny us their important ecosystem services. After understanding the role of bats (or wildlife) in the maintenance and circulation of pathogens and the mechanisms underlying the emergence of zoonotic diseases, wildlife biologists and epidemiologists should work together developing appropriate management plans to control virus circulation. Just then, risks of human infection without causing significant biases against specific animal populations will be minimized.

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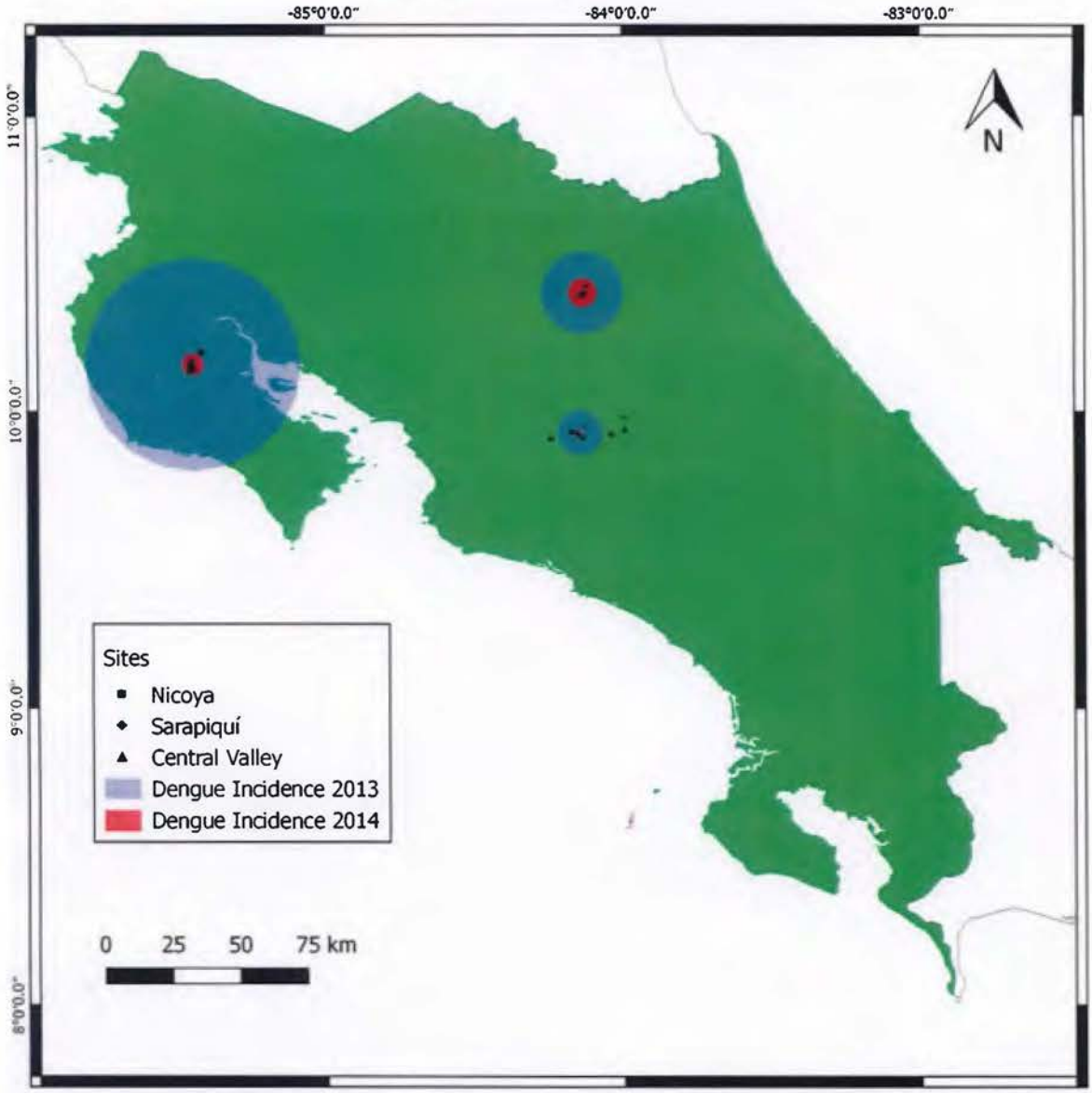

Figure 1. Sampling sites in Costa Rica and their incidence of dengue in the years of the study, 2013 and 2014. Each symbol (■, ♦, ▲) represents a sampled household. These incidence values were retrieved from epidemiological surveillance done by the Ministry of Health. (Referencia: Ministerio de Salud de Costa Rica. Dengue 2015. Análisis de situación de salud. http://www.ministeriodesalud.go.cr/index.php/vigilancia-de-la-salud/analisis-de-situacion-de-salud. Published 2015. January 2016.)

Table 1. Prevalence of DENV RNA obtained by PCR from each serotype (D1-4) in each species of bats captured in the 3 sites of study in **Costa Rica (Nicoya, Sarapiquí and Central Valley) during the dry and rainy** season, 2013 -2014.

Species	Nicoya (N)					Sarapiquí (S)						Central Valley (CV)								Total
	Dry	Rainy			N Total	Dry	Rainy				S Total	Dry			Rainy				CV Total	
	n	n	D2	D4		n	n	D2	D3	D4		n	D1	D2	n	D1	D2	D4		
Balantiopteryx plicata		5			5															5
Eptesicus fuscus															3				3	3
Eumops glaucinus															3	1			3	3
Glossophaga soricina												10		1					10	10
Molossus pretiosus		10	1																	10
Molossus rufus												24		2	30		2**	1**	54	54
Molossus sinaloae	40	42	2	3	82	35	62	3*	2*	3	97	10		2	18			2	28	207
Myotis elegans							1				1									1
Myotis nigricans						2	1				3									3
Rhogeessa io							1				1									1
Rhogeessa bickhami												10	1	1	10	3		1	20	20
Uroderma convexum	1				1															1
Total	41	57	3	3	88	37	65	3	2	3	102	54	1	6	64	4	2	4	118	318

*Two individuals with joint detection of DENV-3 and DENV-2. **One individual with joint detection of DENV-4 and DENV-2

Table 2. Seroprevalence against each dengue serotype obtained from serum diluted 1:20 by $PRNT_{90}$ from bats captured in the 3 sites of study in Costa Rica (Nicoya, Sarapiquí and Central Valley) during the dry and rainy season, 2013 -2014.

Species	Nicoya (N)				N Total	Sarapiquí (S)			S Total	Central Valley (CV)			CV Total	Total
	D1	D2	D3	D4		D1	D2	D3		D1	D2	D3		
Balantiopteryx plicata			1		4									4
Eptesicus fuscus													3	3
Eumops glaucinus													3	3
Glossophaga soricina												1	5	5
Molossus pretiosus		1			8									8
Molossus rufus										2	1	2	35	35
Molossus sinaloae	5		8	2	69	5	7	17	72	1		5	19	160
Myotis elegans									1					1
Myotis nigricans									1					1
Rhogeessa io								1	1					1
Rhogeessa bickami										2	2		19	19
Uroderma convexum					1									1
Total	5	1	9	2	82	5	7	18	75	5	3	8	84	241

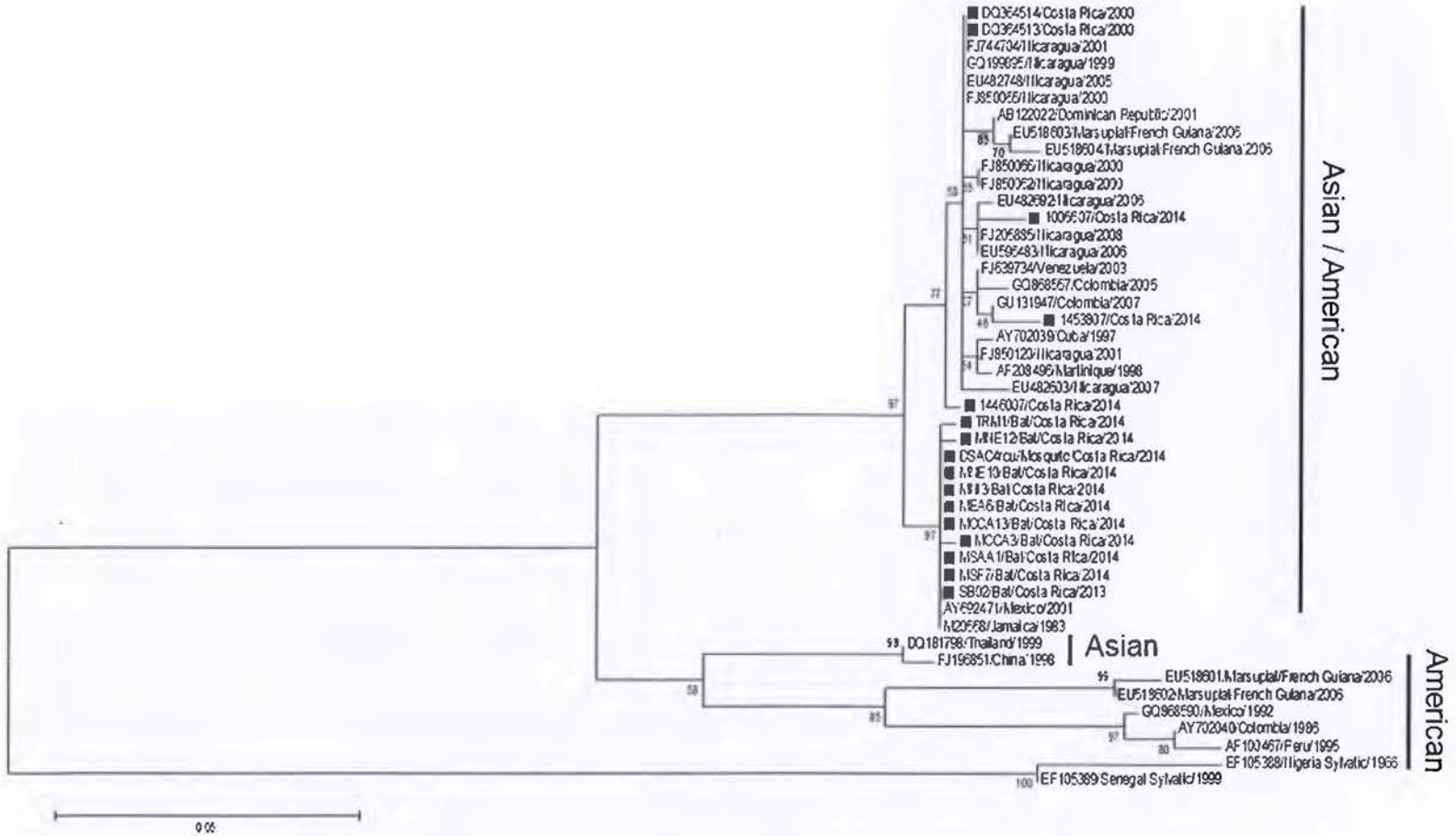

Figure 2. Maximum likelihood tree of 49 DENV-2 C/prM sequences gene sequences (390 bp) including 10 from bats, 1 from a mosquito, and 3 from humans from former human outbreaks in Costa Rica. Black rectangles indicates the strains from Costa Rica. Significant bootstrap values (≥ 70) are indicated at the respecting nodes. The sequences were named according to reference number/country/year of collection or detection.

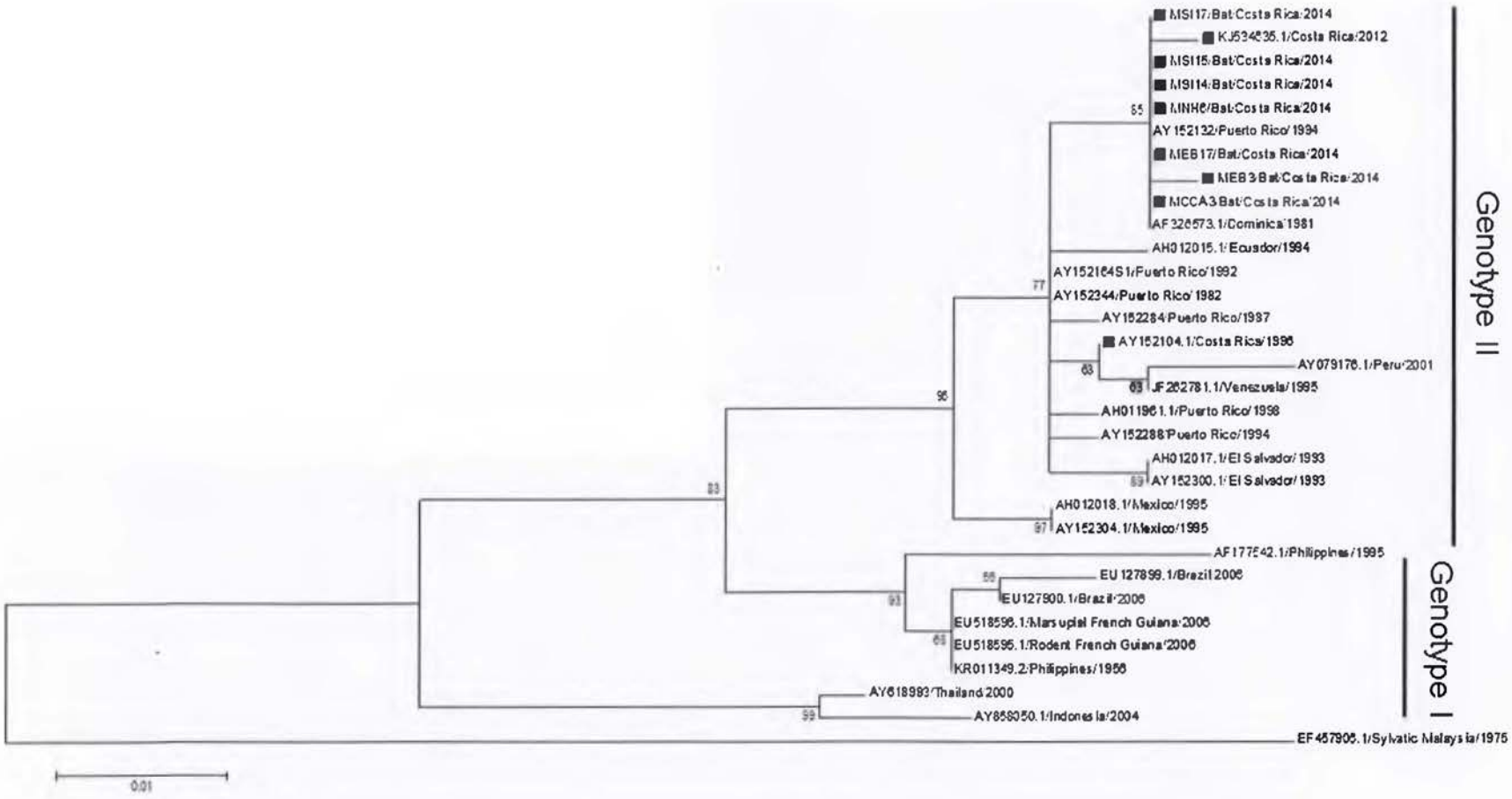

Figure 3. Maximum likelihood tree of 31 DENV-4 C/prM sequences gene sequences (390 bp) including 7 from bats. Black rectangles indicates the strains from Costa Rica. Significant bootstrap values (≥ 70) are indicated at the respecting nodes. The sequences were named according to reference number/country/year of collection or detection.

Apéndices

Apéndice 1.1 Primers utilizados en el estudio

Supplementary Table 1. Primers used in the study.

Primer	Sequence 5'-3'	Genome position	Use	Reference
D1	TCAATATGCTGAAACGCGCGAGAAACCG	134-161	PCR	Lanciotti *et al.* 1992
D2	TTGCACCAACAGTCAATGTCTTCAGGTTC	616-644	PCR	Lanciotti *et al.* 1992
TS1	CGTCTCAGTGATCCGGGGG	568-586	PCR	Lanciotti *et al.* 1992
TS2	CGCCACAAGGGCCATGAACAG	232-252	PCR	Lanciotti *et al.* 1992
TS3	TAACATCATCATGAGACAGAGC	400-421	PCR	Lanciotti *et al.* 1992
TS4	CTCTGTTGTCTTAAACAAGAGA	506-527	PCR	Lanciotti *et al.* 1992
DenS	GGATAGACCAGAGATCCTGCTGT	10615-10636	qPCR	Drosten *et al.* 2002
DenAs	CATTCCATTTTCTGGCGTTC plus CAATCCATCTTGCGGCGCTC	10694-10675	qPCR	Drosten *et al.* 2002
DenP	CAGCATCATTCCAGGCACAG	10656-10675	qPCR probe	Drosten *et al.* 2002
COI_long (f)	AACCACAAAGACATTGGCAC	5934[a]	PCR	Townzen *et al.* 2008
COI_long (r)	AAGAATCAGAATARGTGTTG	6597[a]	PCR	Townzen *et al.* 2008
COI_short (f)	GCAGGAACAGGWTGAACCG	6267[a]	PCR	Townzen *et al.* 2008
COI_short (r)	AATCAGAAYAGGTGTTGGTATAG	6591[a]	PCR	Townzen *et al.* 2008
Cyt *b* (f)	GAGGMCAAATATCATTCTGAGG	15 150[a]	PCR	Townzen *et al.* 2008
Cyt *b* (r)	TAGGGCVAGGACTCCTCCTAGT	15 607[a]	PCR	Townzen *et al.* 2008
D1	TCAATATGCTGAAACGCGCGAGAAACCG	134-161[b]	PCR, Sequencing	Díaz *et al.* 2006
D1-682F	AACCGGYGAACACCGACGAGA	682	Sequencing	Díaz *et al.* 2006
D1-1064F	GAACTCTTGAAGACGGAGGTCACGAA	1064	Sequencing	Díaz *et al.* 2006
D1-1167R	TTGTTCTTCCACCAGTGTAGCCTCTC	1167	Sequencing	Díaz *et al.* 2006
D1-1488F	GCTCACCTAGAACAGGGCTGGACTTT	1488	Sequencing	Díaz *et al.* 2006
D1-1649R	TTCTTTGCATGAGCTGTCTTGAATGT	1649	Sequencing	Díaz *et al.* 2006
D1-1972F	CCAGAATGGGAGATTGATAACA	1972	Sequencing	Díaz *et al.* 2006
D1-2125R	CGGTTGCTTCGAACATTTTCCCTATG	2125	Sequencing	Díaz *et al.* 2006

D1-2726R	ATGGGTTGTGGCCTAATCAT	2726	PCR, Sequencing	Díaz *et al.* 2006
D2-739F	ATGGGATTGGAGACACGAACTGAA	739	Sequencing	Díaz *et al.* 2006
D2-1184F	ATGAAGAGCAGGACAAAAGGTT	1184	Sequencing	Díaz *et al.* 2006
D2-1225R	CCATTTCCCCATCCTCTGTCTAC	1225	Sequencing	Díaz *et al.* 2006
D2-1540F	GAAGACAAAGCTTGGCTGGTG	1540	Sequencing	Díaz *et al.* 2006
D2-1648R	GCATGGGGATTTTTGAARGTGAC	1648	Sequencing	Díaz *et al.* 2006
D2-2028F	GGAGGTTCTGCTTCTATGTTGACT	2028	Sequencing	Díaz *et al.* 2006
D2-2120R	TCAAACATTTGGCCGATRGAACTTC	2120	Sequencing	Díaz *et al.* 2006
D2-2588R	TCTTGTTACTGAGCGGATTC	2588	PCR, Sequencing	Díaz *et al.* 2006
D3-644F	CTTACATCAACATGGGTGACTTAT	644	Sequencing	Díaz *et al.* 2006
D3-1343F	TACACCGTCATCATCACAGTG	1343	Sequencing	Díaz *et al.* 2006
D3-1390R	CTTACATCAACATGGGTGACTTAT	1390	Sequencing	Díaz *et al.* 2006
D3-1671F	AAGAAGTAGTTGTCCTTGGAT	1671	Sequencing	Díaz *et al.* 2006
D3-1953R	CATTGTGAGCTTTCCCTTGTC	1953	Sequencing	Díaz *et al.* 2006
D3-2429R	TTCTTTGCCTTTCCAGTTTAT RT	2429	PCR, Sequencing	Díaz *et al.* 2006
D4-762F	GAGACATGGATGTCATCGGAAGG	762	Sequencing	Díaz *et al.* 2006
D4-1240F	GGGGCAATGGCTGTGGCTTGTT	1240	Sequencing	Díaz *et al.* 2006
D4-1308R	TTGGACCAAATTGCCTGTTATCTT	1308	Sequencing	Díaz *et al.* 2006
D4-1667F	YCATGCCAAGAGACAGGATGTGAC	_1667	Sequencing	Díaz *et al.* 2006
D4-1702R	GCAAGAATGCATGGCTCCTTCCTGAG	1702	Sequencing	Díaz *et al.* 2006
D4-2158R	GAATGGCCATTCGTTTTGCACCTC	2158	Sequencing	Díaz *et al.* 2006
D4-2052F	CCYTTTGGGGACAGCGCTACATA	2052	Sequencing	Díaz *et al.* 2006
D4-2649R	TGTCCTCCTTCCCAGAGAACATAGTT	2649	PCR, Sequencing	Díaz *et al.* 2006
D1L	TAGGTCATTGTGTCCTCACATAACTCTCC	560-588	PCR, sequencing	De Thoisy *et al.* 2009
D2L	CTTGTACGTGATTGTATCTTCACACA	569-594	PCR, sequencing	De Thoisy *et al.* 2009
D3L	TTGTAAGTGACCGTGTCATCACACAT	566-594	PCR, sequencing	De Thoisy *et al.* 2009
D4L	TCCATGGCAATGAGAGTGCATTTGTTGA	533-560	PCR, sequencing	De Thoisy *et al.* 2009

[a]Location on human mtDNA

[b]The priming position of primer D1 in each genome was as follows: type 1: 132; type 2: 134; type 3: 132; type 4: 137.

Apéndice 1.2 Secuencias utilizadas en los análisis filogenéticos

Supplementary Table 2. Information of DENV-2 sequences used in phylogenetic analysis.

DENVs strains in this study	GenBank Accession number	Country	Year	Strain	Genotype
AY702040/Colombia/ 1986	AY702040	Colombia	1986	DENV-2/CO/I348600/1986	American
AF100467/Peru/1995	AF100467	Peru	1995	DENV-2/PE/IQT1797/1995	American
GQ868590/Mexico/ 1992	GQ868590	Mexico	1992	DENV-2/MX/BID-V3356/1992	American
EU518601/Marsupial/ French Guiana/2006	EU518601	French Guiana	2006	DENV-2/FG/B1032/2006	American
EU518602/Marsupial/ French Guiana/2006	EU518602	French Guiana	2006	DENV-2/FG/B1015/2006	American
EU518603/Marsupial/ French Guiana/2006	EU518603	French Guiana	2006	DENV-2/FG/B1010/2006	Asian/ American
EU518604/Marsupial/ French Guiana/2006	EU518604	French Guiana	2006	DENV-2/FG/B1004/2006	Asian/ American
FJ205885/Nicaragua/ 2008	FJ205885	Nicaragua	2008	DENV-2/NI/BID-V1721/2008	Asian/ American
EU596483/Nicaragua/ 2006	EU596483	Nicaragua	2006	DENV-2/NI/BID-V608/2006	Asian/ American
EU482748/Nicaragua/ 2005	EU482748	Nicaragua	2005	DENV-2/NI/BID-V513/2005	Asian/ American
EU482603/Nicaragua/ 2007	EU482603	Nicaragua	2007	DENV-2/NI/BID-V615/2007	Asian/ American
FJ850065/Nicaragua/ 2000	FJ850065	Nicaragua	2000	DENV-2/NI/BID-V2664/2000	Asian/ American
GQ199895/Nicaragua/ 1999	GQ199895	Nicaragua	1999	DENV-2/NI/BID-V2683/1999	Asian/ American
FJ850120/Nicaragua/ 2001	FJ850120	Nicaragua	2001	DENV-2/NI/BID-V2673/2001	Asian/ American
EU482683/Nicaragua/	EU482683	Nicaragua	2006	DENV-2/NI/BID-V570/2006	Asian/

2006					American
EU482692/Nicaragua/ 2006	EU482692	Nicaragua	2006	DENV-2/NI/BID-V580/2006	Asian/ American
FJ744745/Nicaragua/ 2000	FJ744745	Nicaragua	2000	DENV-2/NI/BID-V2362/2000	Asian/ American
FJ744704/Nicaragua/ 2001	FJ744704	Nicaragua	2001	DENV-2/NI/BID-V2361/2001	Asian/ American
FJ850066/Nicaragua/ 2000	FJ850066	Nicaragua	2000	DENV-2/NI/BID-V2665/2000	Asian/ American
FJ850062/Nicaragua/ 2000	FJ850062	Nicaragua	2000	DENV-2/NI/BID-V2659/2000	Asian/ American
FJ639734/Venezuela/ 2003	FJ639734	Venezuela	2003	DENV-2/VE/BID-V2160/2003	Asian/ American
GQ868557/Colombia/ 2005	GQ868557	Colombia	2005	DENV-2/CO/BID-V3373/2005	Asian/ American
GU131947/Colombia/ 2007	GU131947	Colombia	2007	DENV-2/CO/BID-V3374/2007	Asian/ American
DQ364514/Costa Rica/2000	DQ364514	Costa Rica	2000	DENV-2/CR/CR_7945_00/2000	Asian/ American
DQ364513/Costa Rica/2000	DQ364513	Costa Rica	2000	DENV-2/CR/CR_6530_00/2000	Asian/ American
AY702039/Cuba/ 1997	AY702039	Cuba	1997	DENV-2/CU/Cuba205_97/1997	Asian/ American
AF208496/Martinique/ 1998	AF208496	Martinique	1998	DENV-2/MTQ/H_IMTSSA_MART_98-703/1998	Asian/ American
AB122022/Dominican Republic/2001	AB122022	Dominican Republic	2001	DENV-2/DOM/DR59_01/2001	Asian/ American
AY692471/Mexico/ 2001	AY692471	Mexico	2001	DENV-2/MX/HUAT12/2001	Asian/ American
M20558/Jamaica/ 1983	M20558	Jamaica	1983	DENV-2/JAM/Jamaica_N.1409/1983	Asian/ -American
DQ181798/Thailand/ 1999	DQ181798	Thailand	1999	DENV-2/TH/ThD2_0055_99/1999	Asian
FJ196851/China/1998	FJ196851	China	1998	DENV-2/CH/GD08_98/1998	Asian
EF105389/Senegal	EF105389	Senegal	1999	DENV-2/SN/Dak Ar 141069/1999	Sylvatic

Sylvatic/1999					
EF105388/Nigeria Sylvatic/1966	EF105388	Nigeria	1966	DENV-2/NG/IBH11664/1966	Sylvatic
1006607/Costa Rica/2007		Costa Rica	2007		
1446007/Costa Rica/2007		Costa Rica	2007		
1453807/Costa Rica/2007		Costa Rica	2007		
DSAC4r/Moquito/Costa Rica/2014		Costa Rica	2014		
MCCA3/Bat/Costa Rica/2014		Costa Rica	2014		
MCCA13/Bat/Costa Rica/2014		Costa Rica	2014		
MEA6/Bat/Costa Rica/2014		Costa Rica	2014		
MNE10/Bat/Costa Rica/2014		Costa Rica	2014		
MNE12/Bat/Costa Rica/2014		Costa Rica	2014		
MNI3/Bat/Costa Rica/2014		Costa Rica	2014		
MSAA1/Bat/Costa Rica/2014		Costa Rica	2014		
MSF7/Bat/Costa Rica/2014		Costa Rica	2014		
SB02/Bat/Costa Rica/2013		Costa Rica	2013		
TRM1/Bat/Costa Rica/2014		Costa Rica	2014		

Supplementary Table 3. Information of DENV-4 sequences used in phylogenetic analysis.

DENVs strains in this study	GenBank Accession number	Country	Year	Strain	Genotype
KR011349.2/Philippines/ 1956	KR011349.2	Philippines	1956	DENV-4/PH/H241/1956	Genotype I
EU127899.1/Brazil/2006	EU127899.1	Brazil	2006	DENV-4/BR/AM750/2006	Genotype I
AF177542.1/Philippines/ 1995	AF177542.1	Philippines	1995	DENV-4/PH/Mindanao_BDJ_1995/ 1995	Genotype I
EU518596.1/Marsupial French Guiana/2006	EU518596.1	French Guiana	2006	DENV-4/FG/B1008/2006	Genotype I
EU518595.1/Rodent French Guiana/2006	EU518595.1	French Guiana	2006	DENV-4/FG/B1106/2006	Genotype I
AY079176.1/Peru/2001	AY079176.1	Peru	2001	DENV-4/PR/Sullana-Peru_6682-01/2001	Genotype I
AF326573.1/Dominica/ 1981	AF326573.1	Dominican Republic	1981	DENV-4/DOM/814669/1981	Genotype II
AH012015.1/Ecuador/ 1994	AH012015.1	Ecuador	1994	DENV-4/EC/D4.109_1994EC/1994	Genotype II
AY152288/Puerto Rico/1994	AY152288	Puerto Rico	1994	DENV-4/PR/D4.88_1994/1994	Genotype II
AY152284/Puerto Rico/1987	AY152284	Puerto Rico	1987	DENV-4/PR/D4.9_1987/1987	Genotype II
AY152164S1/Puerto Rico/1992	AY152164S1	Puerto Rico	1992	DENV-4/PR/D4.25_1992/1992	Genotype II
AH011961.1/Puerto Rico/1998	AH011961.1	Puerto Rico	1998	DENV-4/PR/D4.48_1998/1998	Genotype II
JF262781.1/Venezuela/ 1995	JF262781.1	Venezuela	1995	DENV-4/VE/INH6412/1995	Genotype II
AY152344/PuertoRico/ 1982	AY152344	PuertoRico	1982	DENV-4/PR/D4M.3_1982/1982	Genotype II
EU127900.1/Brazil/2006	EU127900.1	Brazil	2006	DENV-4/BR/AM1619/2006	Genotype II
AY152132/PuertoRico/ 1994	AY152132	PuertoRico	1994	DENV-4/PR/D4.77_1994/1994	Genotype II
AH012018.1/Mexico/	AH012018.1	Mexico	1995	DENV-4/MX/D4.111_1995MX/199	Genotype II

1995				5	
AY618993/Thailand/2000	AY618993	Thailand	2000	DENV-4/TH/ThD4_0734_00/2000	Genotype II
AY858050.1/Indonesia/2004	AY858050.1	Indonesia	2004	DENV-4/ID/SW38i/2004	Genotype II
AY152304.1/Mexico/1995	AY152304.1	Mexico	1995	DENV-4/MX/D4.111_1995MX/1995	Genotype II
EU522110.1/French Guiana/2005	EU522110.1	French Guiana	2005	DENV-4/FG/2005	Genotype II
KJ534635.1/Costa Rica/2012	KJ534635.1	Costa Rica	2012	DENV-4/CR/P30/2012	Genotype II
AY152104.1/Costa Rica/1996	AY152104.1	Costa Rica	1996	DENV-4/CR/D4.108_1996CR/1996	Genotype II
EF457906.1/Sylvatic Malaysia/1975	EF457906.1	Sylvatic Malaysia	1975	DENV-4/MY/P75-215/1975	Sylvatic
MCCA3/Bat/Costa Rica/2014		Costa Rica	2014		
MEB3/Bat/Costa Rica/2014		Costa Rica	2014		
MEB17/Bat/Costa Rica/2014		Costa Rica	2014		
MNB8/Bat/Costa Rica/2014		Costa Rica	2014		
MNH6/Bat/Costa Rica/2014		Costa Rica	2014		
MSI14/Bat/Costa Rica/2014		Costa Rica	2014		
MSI15/Bat/Costa Rica/2014		Costa Rica	2014		
MSI17/Bat/Costa Rica/2014		Costa Rica	2014		

Apéndice 1.3 Protocolos empleados en el estudio

Protocolo de extracción de ARN con Trizol

1. 100µL de la muestra + 750µL de Trizol frio
2. Vortex e incubar a temperatura ambiente (TA) por 5'
3. Agregar 200µL de cloroformo frio
4. Vortex e incubar a TA por 5'
5. Centrifugar a 12000rpm por 15'
6. Pasar sobrenadante a tubo limpio que contenga 3µL de Glycoblue.
7. Precipitar agregando 500µL de isopropanol
8. Incubar a TA por 10'
9. Centrifugar a 12000rpm por 10'
10. Decantar sobrenadante
11. Lavar pellet de ARN con 1mL de etanol frio al 75%
12. Incubar a TA por 2-4'
13. Centrifugar a 10000rpm por 5'
14. Decantar y dejar secar el etanol
15. Resuspender el ARN en 30µL de DEPC H_20

Protocolo de retrotranscripción de ARN

10µL ARN + 1µL DMSO + 1µL Random Primers (o primers específicos)

Termociclador: 5-10' a 65°C

Enfriar en hielo y agregar 8µL del Master Mix (MM) a cada muestra:

MM	1x
5x Reaction buffer	4µL
Ribolock RNAse inhibitor	1µL
10MM dNTP mix	2µL
Revertaid H minus MMolv trans	1µL
Vol. Final =	20µL

Mezclar y centrifugar

Termociclador:	25°C	5'
	42°C	60'
	70°C	5'
	4°C	∞

Protocolo de PCR Dengue - Lanciotti *et al.* 1992

Primers a 10µM

- 1° PCR

Master Mix	1x
Toptaq	6,25µL
D1	0,25µL
D2	0,25µL
H_2O	3,25µL
	10µL + 2,5µL de ADNc

Termociclador:	94°C	10'
	x 30 ciclos:	
	94°C	30''
	55°C	30''
	72°C	30''
	72°C	10'
	4°C	∞

Diluir producto del 1°PCR 1:100

- 2°PCR – Anidado

Master Mix	1x
Multiplex	6,25µL
Q	1,25µL
H_2O	2,75µL
D1	0,25µL
TS1	0,25µL
TS2	0,25µL
TS3	0,25µL
TS4	0,25µL
	11,5µL + 1µL del producto del 1°PCR diluido

Termociclador: 94°C 5'

x 30 ciclos:

94°C 30''

55°C 1'

72°C 2'

72°C 7'

4°C ∞

Tamaños: D1= 482pb

D2= 119pb

D3= 250pb

D4= 389pb

Protocolo de PCR Secuenciación Dengue – Díaz *et al.* 2006

Master Mix	1x
Toptaq	25µL
D1	1µL
Primer específico R	1µL
H_2O	13µL
	40µL + 10µL ADNc

Primer serotipo específico: D1-2726R
D2-2588R
D3-2429R
D4-2649R

Tamaño: aprox. 2474 – 2577 pb

Termociclador:

Para DENV1, DENV2 y DENV4:		Para DENV3:	
95°C	5'	95°C	5'
x 10 ciclos:		x 10 ciclos:	
95°C	45''	95°C	45''
54°C	2'	51°C	2'
68°C	2'	68°C	2'
x 25:		x 25:	
95°C	45''	95°C	45''
65°C	2'	60°C	2'
68°C	3'	68°C	3'
4°C	∞	4°C	∞

Protocolo de PCR COI_short, COI_long y cyt b

Master Mix	1x
Toptaq	12.5µL
Primer F	0.5µL
Primer R	0.5µL
H2O	6.5µL
	20µL + 5µL de ADN

Termociclador:	95°C	1'
	x 35 ciclos:	
	95°C	30"
	50°C	50"
	72°C	1'
	72°C	5'
	4°C	∞

Tamaños: COI_short= 300-400pb

COI_long= 600-700pb

Cyt b= 400-500pb

Protocolo de PCR secuenciación De Thoisy *et al.* 2009

Se parte del producto del 1°PCR de Lanciotti diluido 1:100

Master Mix	1x
Toptaq	12.5µL
D1	0.5µL
Primer R (D1L-D4L)	0.5µL
H2O	6.5µL
	20µL + 5µL de producto de 1° PCR diluido 1:100

Termociclador:

	94°C	5'
x 25 ciclos:		
	94°C	30"
	52°C	90"
	72°C	1'
72°C	10'	
4°C	∞	

Tamaño:

D1L= 457pb

D2L= 461pb

D3L= 460pb

D4L= 424pb

Protocolo Exosap

Preparación de 200µL:	Exonucleasa	2µL
	Buffer Exo1	20µL
	Fosfatasa alcalina	20µL
	Buffer 10 (Fasap)	20µL
	ALN	138µL
		200µL

De esta mezcla:

4µL Exosap + 10µL producto PCR → incubar a 37°C por 30' → inactivación a 95°C por 5' → 4°C ∞

Protocolo de qPCR DENV Drosten *et al.* 2002

Master Mix	1x	
Agua	2,2µL	
2x Rxn Mix SSCIII	12,5µL	
Den-S	0,5µL	
Den-As		1,0µL
Den-P	0,5µL	
BSA	1,0µL	
$MgSO_4$		1,3µL
SSCIII/Platinum Taq	1.0µL	
	20µL + 5µL ARN	
StepOne RT-PCR conditions:		45°C 30'
	95°C 5`	
	X 45:	
		95°C 5''
		57°C 35''

Protocolo de microneutralización de dengue

En placas de 96 pozos. Recubrir con células Vero o MK2.

Tratamientos (por duplicado):

1. Células
2. Células + virus
3. Células + virus + suero humano positivo en dilución 1:20
4. Células + virus + suero humano positivo en dilución 1:50
5. Células + virus + suero humano negativo a 1:20
6. Células + virus + suero murciélago positivo en dilución 1:20
7. Células + virus + suero murciélago positivo en dilución 1:50
8. Células + suero murciélago

Se diluye el suero y el virus a al MOI deseado en MEM al 2% → Incubar a 37°C por 1h

Remover medio de cultivo de cada pozo y agregar 50µL de cada tratamiento a cada pozo → Incubar a 37°C por 1h30'' → remover tratamiento → agregar 100µL de medio de placas (MEM con metilcelulosa al 1,5%).

Incubar por 3 días a 37°C

Fijación con metanol:

1. Remover sobrenadante de las células
2. Agregar 50µL de metanol frío (en -20°C) a cada pozo
3. Dejar células a -20°C por 12h o toda la noche

Tinción:

a. Preparar 10mL de PBS 1x no estéril con leche descremada al 1% (0,1g de leche + 10mL de PBS)
b. Diluir anticuerpo primario [MouseαD1,2,3,4 CeneTex] (o anticuerpo de Hennessy para DENV4) al 1:400. (calcular al menos 100µL más del que se necesita)
c. Remover metanol: dejar secar 5' y hacer 2 lavados con 100µL de PBS 1x no estéril
d. Recubrir con 50µL de anticuerpo primario cada pozo → incubar 30' a temperatura ambiente (TA) → remover → 3 lavados con 100µL de PBS 1x no estéril
e. Diluir anticuerpo secundario [goatαmouse con peroxidasa] al 1:200

f. Recubrir con 50µL de anticuerpo secundario cada pozo → incubar 1h a TA → remover → 3 lavados con 100µL de PBS 1x no estéril
g. Preparar reactivo peroxidasa (en el momento): Diluir 1:5 el AEC en Buffer ACO-/ACOH pH 5-5,5 + 10µL de peróxido al 30%.
    a. Preparación AEC: 1 pastilla de 3-amino-9-ethylcarbazole en 4mL de N,N-Dimethyl-formamide. Se proteje de la luz
h. Agregar 50µL a cada pozo → incubar 30' a TA → remover → 2 lavados con 100µL de PBS 1x no estéril → agrego 100µL de PBS 1x no estéril
i. Conteo de placas en el estereoscopio