UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

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(**ESTUDIO DE LAS ESPECIES DE *BARTONELLA*, *RICKETTSIA* Y *LEPTOSPIRA* ASOCIADAS CON ROEDORES Y ECTOPARÁSITOS DE MÉXICO: DESARROLLO DIAGNÓSTICO Y ANÁLISIS DE PREVALENCIA**)

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Resumen

Las enfermedades emergentes son un problema de salud pública que se ha agudizado durante las últimas décadas. Cerca del 65%, de los patógenos que afectan a las poblaciones humanas son de origen zoonótico y un 12% de éstos son reemergentes. En particular aquellas causantes del Síndrome Febril Hemorrágico Agudo, resultan un reto para la clínica debido a la poca especificidad del cuadro clínico y la falta de herramientas para su diagnóstico diferencial. Dentro de estas patologías encontramos a la Bartonelosis, Leptospirosis y Rickettsiosis, enfermedades asociadas con la presencia de fauna silvestre, cuyos agentes etiológicos exhiben ciclos de vida complejos con múltiples hospederos y vectores que pueden entrar en contacto con poblaciones humanas y generar brotes con una elevada mortalidad. En consecuenica, resulta fundamental el monitoreo de las especies causantes de estas patologías en fauna silvestre, para identificar zonas de riesgo.

Por tal motivo el objetivo de este estudio fue identificar la presencia, diversidad y prevalencia de las especies de *Bartonella*, *Leptospira* y *Rickettsia* en mamíferos pequeños y ectoparásitos de tres estados (Hidalgo, Jalisco y San Luis Potosí) con reportes previos de casos humanos de estas patologías.

Se realizaron múltiples colectas donde se recuperaron diversas especies de mamíferos y sus respectivos ectoparásitos (garrapatas, piojos y pulgas). Los hospederos pequeños fueron sacrificados y se extrajeron muestras de bazo, hígado y oreja, así como sus ectoparásitos asociados, las cuales se examinaron para identificar la presencia de los patógenos mediante la amplificación de varios fragmentos de los genes *16S-rRNA*, *gltA*, *ompA*, *ompB*. Paralelamente se realizó la identificación morfológica de los hospederos y ectoparásitos, y la caracterización molecular de estos últimos mediante la amplificación de los genes mitocondriales *COI* y *16S-rRNA*.

Registramos por primera vez una especie de *Bartonella* (*B. vinsonii*) en piojos chupadores [*Hoplopleura hirsuta*], los cuales podrían fungir como vectores potenciales de este patógeno en poblaciones de la rata algodonera (*Sigmodon hispidus*) en Norteamérica. Adicionalmente se registraron dos nuevas especies de pulgas [*Peromyscopsylla hesperomys* y *Plusaetis mathesoni*] infectadas por otro linaje de *B. vinsonii*, también detectado en el hospedero de las mismas, el ratón de Orizaba (*Peromyscus beatae*). Es importante realzar que registramos por primera vez en México a un agente zoonótico declarado (*Bartonella elizabethae*) en ejemplares de rata noruega (*Rattus norvergicus*).

Por otro lado se identificó la epidemiología y distribución de la leptospirosis humana en el país. Nuestros resultados permitieron determinar que esta enfermedad considerada como tropical se encuentra ampliamente diseminada en el país, registrándose en 27 estados y concentrando la mayoría de los casos durante la temporada de lluvias en focos del Centro, Oeste y Sureste del país.

En el caso de *Rickettsia* brindamos el primer registro de *Rickettsia amblyommi*, un miembro del grupo de las Fiebres Manchadas, en garrapatas de la especie *Amblyomma mixtum* colectadas de personas en la Costa Oeste de México donde solo existía el registro de *Rickettsia rickettsii*. Adicionalmente, se demostró por primera vez la presencia de *Rickettsia typhi* en *R. norvergicus,* pudiendo incriminar a esta especie como potencial reservorio en un foco de tifo murino en el estado de Hidalgo.

Los resultados del presente trabajo representan el primer registro de varios patógenos zoonóticos confirmados que pueden causar Endocarditis, Tifo y Fiebre Manchada en múltiples especies de hospederos y vectores para México, lo que pone de manifiesto la importancia del establecimiento de un sistema de vigilancia epidemiológico en fauna silvestre.

Introducción

Generalidades de las zoonosis

Las enfermedades emergentes y reemergentes constituyen un problema de salud pública a escala global. Por definición una enfermedad emergente es aquella causada por agentes etiológicos previamente no descritos o aquellos que han aparecido por primera vez en una población, con rápido aumento de su incidencia y área de distribución, como lo son el SARS Co-V, el virus del Ébola o las Neoehrlichias (Bermejo et al. 2006; Mackey et al. 2014; Vouga y Greub, 2016).

En contraparte, las enfermedades reemergentes son aquellas causadas por agentes etiológicos que previamente estuvieron bajo programas de control/eliminación que reaparecen o incrementan su prevalencia en un área geográfica determinada. Esto se debe a múltiples factores entre los que se encuentran la falta de monitoreo, la emergencia de condiciones particulares de inmunosupresión en los hospederos (*eg*. VIH o pacientes que reciben quimioterapia) o hacinamiento como en el caso de *Mycobacterium tuberculosis*, *Vibrio cholerae* o las diversas especies del género *Leishmania* (Gomez-Lus et al. 2000; Palmer, 2007; Abera et al. 2016).

La mayoría de las enfermedades emergentes y reemergentes se encuentran agrupadas en la categoría de zoonosis. La Organización Mundial de la Salud (OMS) estableció en la década de los 90s este término que hace referencia a cualquier enfermedad o infección causada por cualquier tipo de agente infeccioso (virus, bacterias, hongos, parásitos y aquellos no convencionales) que pueden transmitirse de animales vertebrados al hombre y viceversa en forma directa o a través de reservorios, vectores o alimentos (Acha y Szyfres, 2003; Rabozzi et al. 2012).

Actualmente se calcula que un 65% de los padecimientos infecciosos que aquejan a las poblaciones humanas son de origen zoonótico emergente o reemergente; reconociéndose alrededor de 900 especies de patógenos que incluyen virus y priones (217), bacterias (538), hongos (307), protozoarios (66) y helmintos (287) (Taylor et al. 2001; Ecker et al. 2005; Karesh et al. 2012).

Muchos de estos agentes al colonizar los tejidos del hospedero humano se reproducen rápidamente, degradando las células sanas y produciendo toxinas, las cuales interfieren con las funciones celulares del hospedero y generan manifestaciones clínicas comunes como: adenopatía, exantema, fiebre, hemorragias, y/o ictericia, por lo cual se

agrupan dentro del denominado Síndrome Febril Hemorrágico Agudo (SFHA). Dentro de esta categoría se encuentran múltiples enfermedades virales (Influenza, Dengue, Chikungunya, Zika, Fiebre Amarilla, Hantavirus, Arenavirus, Hepatitis), parasitarias (Malaria, Toxoplasmosis, Absceso hepático amebiano) y bacterianas (Fiebre tifoidea, Salmonelosis, Rickettsiosis, Leptospirosis y Bartonelosis) las cuales pueden generar falla sistémica y ocasionar una elevada letalidad, lo cual las convierte en padecimientos de relevancia para la salud pública (Bottieau et al. 2006; Iralú et al. 2006; Lorenzi et al. 2013; Cortés et al. 2016).

Estos últimos tres padecimientos engloban a un conjunto de afecciones causadas por diversas especies de bacterias, asociadas con múltiples especies de hospederos vertebrados (en especial roedores y murciélagos) y artrópodos hematófagos (garrapatas, piojos y pulgas).

Agentes etiológicos

Leptospira y Leptospirosis

La leptospirosis es la zoonosis bacteriana más ampliamente distribuida en el mundo, causada por espiroquetas con extremos en gancho pertenecientes al género *Leptospira*, las cuales habitan ambientes lénticos (particularmente pantanales) y cuyas especies patógenas se encuentran asociadas con alrededor de 160 especies de mamíferos silvestres y domésticos. De manera clásica se reconocía la existencia de dos especies: *Leptospira biflexa* (saprófita) y *Leptospira interrogans* (patógena), las cuales a su vez se clasifican por sus diferencias antigénicas en más de 250 serovares pertenecientes a 23 serogrupos (Levett, 2001; Bharti et al. 2003; Adler y de la Peña-Moctezuma, 2010).

Sin embargo, con el advenimiento de las técnicas de biología molecular y la implementación de la secuenciación del gen 16s ribosomal, Levett (2015) reconoce la existencia de 21 genoespecies pertenecientes a tres grupos delimitados por su capacidad de infectar hospederos vertebrados: las no patógenas (eg. *L. biflexa*, *L. idonoii*, *L. meyeri*), patógenos facultativos *(L. broomii*, *L. fainei*, *L. wolffii*) y las patógenas (*L. alexanderi*, *L. alstonii*, *L. borgpetersenii*, *L. interrogans*) (Fig. 1).

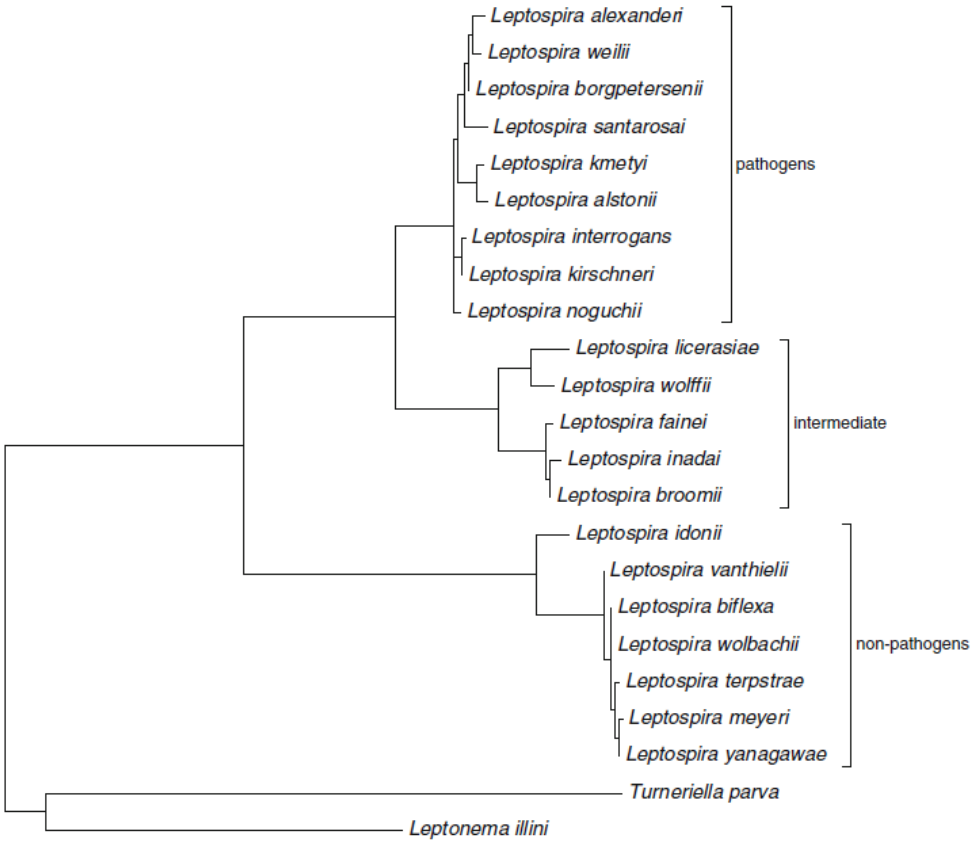

Figura 1. Filogenia de las especies del género *Leptospira* (Tomado de Levett, 2015)

El ciclo de vida de las especies de leptospiras patógenas involucra la presencia de múltiples hospederos mamíferos en los cuales invaden y habitan los túbulos contorneados del riñón. Los reservorios naturales son los roedores, aunque los artiodáctilos, carnívoros, y quirópteros pueden fungir como intermediarios. Los seres humanos son hospederos accidentales en el ciclo de vida natural de la bacteria, y resultan infectados por contacto directo con la orina de animales infectados o indirecto mediante agua y/o lodazales contaminados con dicha orina (Babudieri, 1958; Bunnell et al. 2000; Vado-Solís et al. 2002; Cantu et al. 2008; Espinosa-Martínez et al. 2015) (Fig. 2).

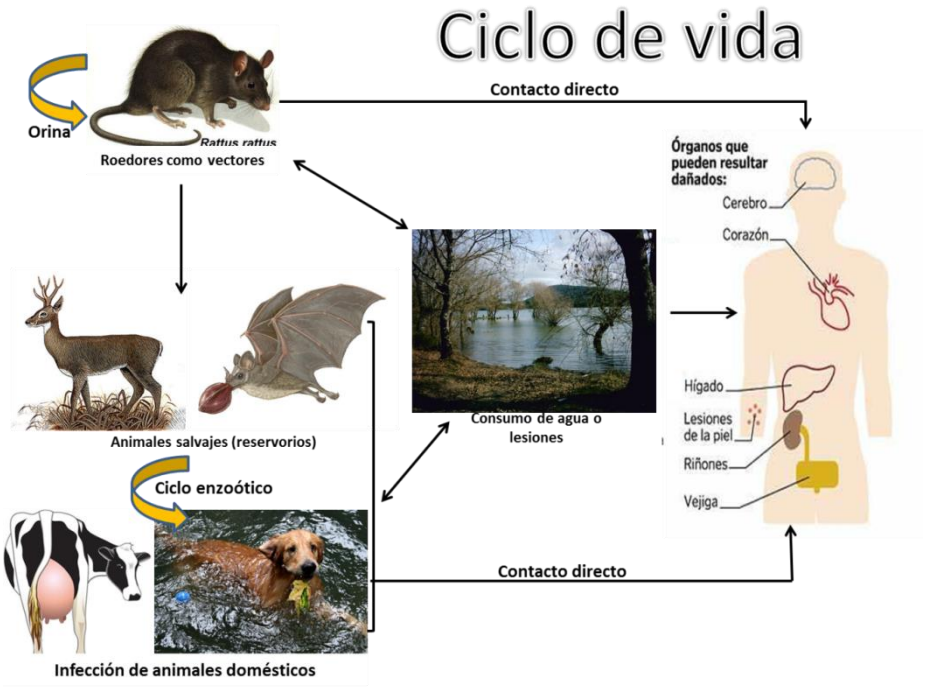

Figura 2. Ciclo de vida de las especies patógenas del género *Leptospira*

Las espiroquetas ingresan al torrente sanguíneo a través de las mucosas o abrasiones en la piel, provocando una bacteremia generalizada e invadiendo todos los tejidos, incluyendo ojos y cerebro, lo cual deriva en la presencia de una amplia gama de signos y síntomas. Sin embargo de manera clásica, se reconoce la presencia de dos fases: la septicémica (durante los primeros siete días de la infección) donde se presenta artralgia, cefalea, mialgia y uveítis y al cabo de uno a tres días una aparente mejoría antes de la recurrencia de síntomas. Posteriormente se desarrolla la segunda fase, también denominada como inmunitaria, la cual se caracteriza por la presencia de alteraciones sistémicas con signos hemorrágicos, manifestaciones cardiacas, hepáticas y renales también reconocidas en su conjunto como enfermedad de Weil, la principal causa de mortalidad en esta patología. A pesar de ello, algunas personas infectadas no muestran síntomas, cursando con una infección subclínica que se resuelve al cabo de unos días de manera espontánea (WHO, 1999, Bharti et al. 2003; Adler y de la Peña-Moctezuma, 2010).

El diagnóstico diferencial depende de la fase en la que se encuentra el paciente, sugiriéndose el uso de diversos protocolos de la Reacción en Cadena de la Polimerasa (PCR) punto final y tiempo real para el diagnóstico de la fase septicémica mediante la

amplificación de fragmentos de los genes *flaB*, *lip32*, *rrs* y *secY* (Gravekamp et al. 1993; Zuerner et al. 2000).

Para la fase inmunitaria, el diagnóstico se basa en la Microaglutinación en placa (MAT), considerado como el estándar de oro, el cual consiste en retar el suero del paciente y/o el animal sospechoso con leptospiras vivas procedentes de cultivo axénico. El panel de diagnóstico dependerá de la implementación de vigilancia epidemiológica previa con el objetivo de identificar los principales genotipos circulantes en la zona, para obtener la mayor representatividad de los organismos circulantes en la zona e incrementar la eficacia del diagnóstico. Otras pruebas serológicas recomendadas son el Ensayo por inmunoadsorción ligado a enzimas (ELISA) y la Inmunofluorescencia, ambas implementadas para la detección de anticuerpos del isotipo IgM anti-leptospira (WHO, 1999; SSA, 2000; Levett, 2001; Bharti et al. 2003).

Existen diversos esquemas quimioterapéuticos utilizados actualmente para el tratamiento del paciente con leptospirosis entre ellos: penicilinas, tetraciclinas, estreptomicina y cloranfenicol, sin embargo, el antibiótico por elección es la doxiciclina (WHO, 1999; SSA, 2000).

Rickettsia y Rickettsiosis

Las Rickettsiosis son un conjunto de enfermedades zoonóticas emergentes causadas por bacterias intracelulares obligadas del género *Rickettsia*. Este taxa se encuentra compuesto por cocobacilos gram-negativos, que son transmitidas por diferentes grupos de artrópodos hematófagos tales como garrapatas, piojos y pulgas. Actualmente se reconoce la existencia de 26 especies las cuales se clasifican en cuatro grandes grupos: el grupo ancestral (*R. bellii*), el grupo intermedio (*R. canadensis* y *R. monteroi*), el grupo de los Tifos [TG] (*R. prowazekii* y *R. typhi*) y el grupo de las Fiebres Manchadas [SGF] (*eg*. *R. conorii, R. massiliae, R. rickettsii* y *R. parkeri*) (Fournier et al. 2003; Fournier y Raoult, 2009; Perlman et al. 2006; Mehrej et al. 2014; Sánchez-Montes et al. 2016) (Fig. 3).

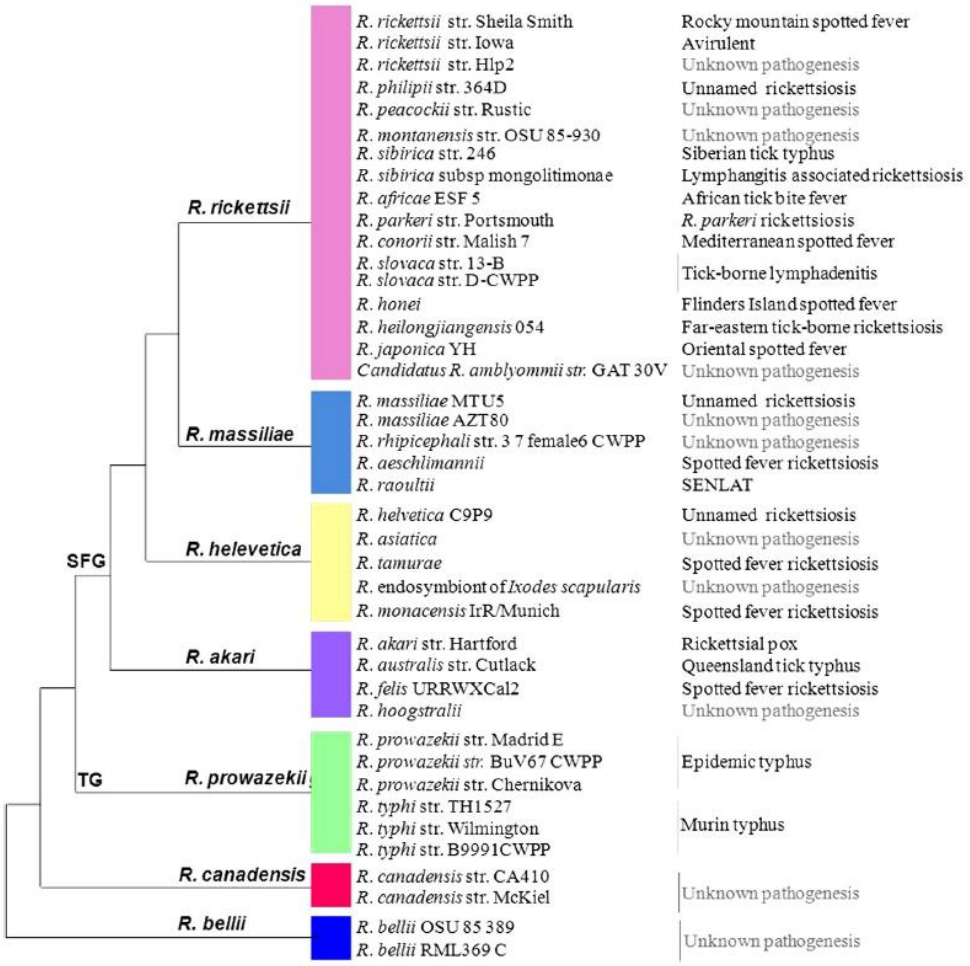

Figura 3. Filogenia de las especies del género Rickettsia (Tomado de Mehrej et al. 2014)

Estas especies se encuentran asociadas con invertebrados no hematófagos, como los psocodeos**,** las sanguijuelas, las hidras y las amebas y en mayor proporción con artrópodos hematófagos tales como garrapatas (especies del SFG), piojos y pulgas (especies del TG) (Dykova et al. 2003; Murray et al. 2016), los cuales adquieren estos patógenos mediante cuatro vías: 1) trasmisión horizontal, cuando un artrópodo no infectado se alimenta de un hospedero infectado y adquiere la bacteria; 2) transestadial, cuando el artrópodo adquiere la bacteria de manera horizontal y esta se mantiene y transmite a través de las restantes fases del ciclo de vida, es decir, de larva a ninfa o de ninfa a adulto; 3) transovárica, en la que el patógeno pasa del progenitor femenino a la descendencia durante la formación del huevo y 4) el "*co-feeding*", el cual se presenta cuando varios artrópodos se alimentan del mismo

hospedero, al mismo tiempo y de manera muy cercana coincidiendo con la presencia del microorganismo en el sitio de la picadura (Brown et al. 2015; Mehrej et al. 2014).

Múltiples especies de vertebrados terrestres participan en el ciclo de vida de las rickettsias, sin embargo, se considera que los roedores son los reservorios por excelencia, debido a que son hospederos naturales de los grupos de ectoparásitos que funcionan como vectores (Burri et al. 2014; Hornok et al. 2014; Laudisoit et al. 2014; Panti-May et al. 2015). El ser humano es un hospedero accidental y la transmisión se presenta cuando los artrópodos infectados se alimentan o cuando el humano esparce en abrasiones de la piel (al rascarse) o inhala las heces infectadas del vector (Faccini-Martínez et al. 2014).

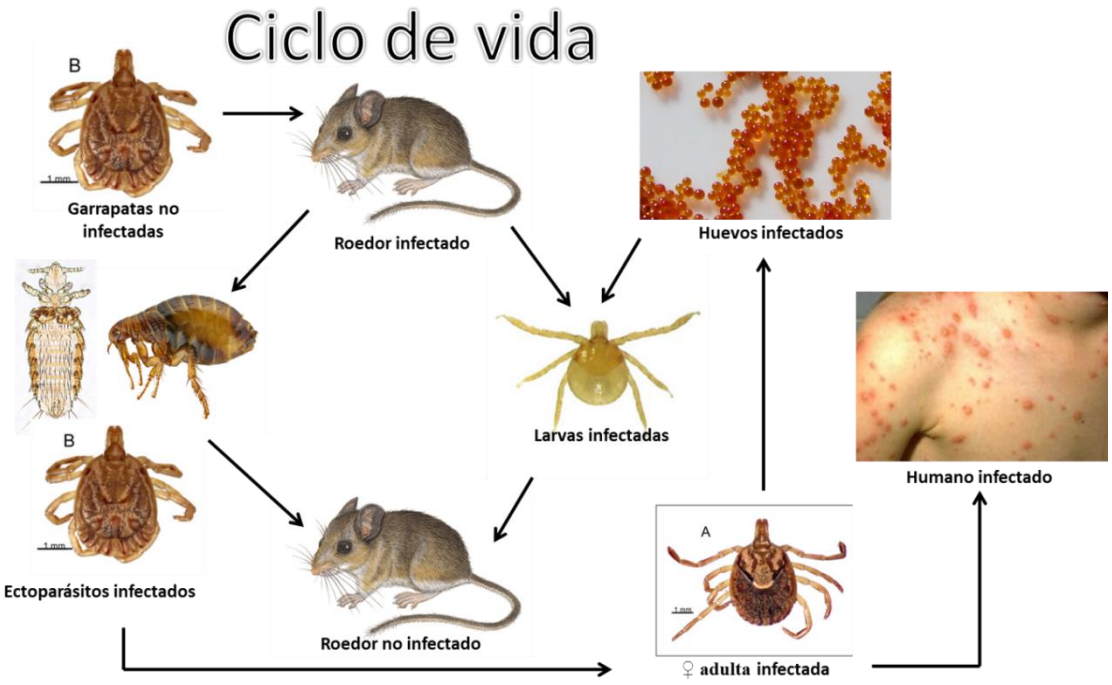

Figura 4. Ciclo de vida de las especies del género *Rickettsia*

Desde el punto de vista clínico, las rickettsiosis pueden dividirse en dos grandes grupos, los Tifos y las Fiebres Manchadas. En general ambas patologías se manifiestan abruptamente con síntomas gripales no específicos que incluyen: cefalea intensa, congestión conjuntival, fiebre, náusea, vómito, dolor abdominal, mialgias y ocasionalmente exantema y fotofobia. También se han reportado signos de meningitis aséptica con confusión mental y rigidez del cuello. Esta fase dura generalmente de cuatro a nueve días.

Sin embargo de manera general se considera la presencia de un exantema centrípeto en el caso de los Tifos y exantema centrífugo, con presencia de escara en el sitio de inoculación del patógeno (donde se adhirió el vector) en las Fiebres Manchadas. En un 5 al 30% de los casos los pacientes desarrollan vasculitis, que conlleva a falla orgánica múltiple, especialmente hepática, respiratoria, renal y nerviosa (Brouqui et al. 2004; Faccini-Martínez et al. 2014; Portillo et al. 2015).

El diagnóstico se realiza mediante la Reacción en Cadena de la Polimerasa en Tiempo Real (rt-PCR) mediante la amplificación de los genes *gltA, ompA* y *htrA* en muestras de sangre, líquido cefalorraquídeo, biopsias de escaras y ectoparásitos. Sin embargo el estándar de oro es la Inmunofluorescencia Indirecta, la cual solo es aplicable a partir de los 7 a 10 días la infección, periodo a partir del cual se desarrollan anticuerpos detectables (Kato et al. 2013; McQuiston et al. 2014; Biggs et al. 2016; Kato et al. 2016).

Con diagnóstico temprano y tratamiento oportuno con doxiciclina, el pronóstico de la mayoría de los pacientes es alentador. Sin embargo, la mortalidad puede ser elevada (50%) en pacientes con retraso en el diagnóstico, antibioterapia inadecuada (uso de sulfas) y presencia de otros padecimientos crónicos (diabetes, obesidad, etc) (Faccini-Martínez et al. 2014; Portillo et al. 2015).

Bartonella y Bartonelosis

Las bartonelosis son un conjunto de enfermedades emergentes y reemergentes causadas por al menos 20 especies de cocobacilos, gram-negativos, de crecimiento intracelular facultativo lento del género *Bartonella*. Una de las características de dichas infecciones es que una misma especie puede causar un amplio espectro de manifestaciones clínicas (*eg*. *Bartonella bacilliformis*), lo cual las vuelve un reto para el diagnóstico clínico (Breitschwerdt y Kordick 2000; Kaiser et al. 2011; Regier et al. 2016).

Con el advenimiento de la biología molecular el número de especies se ha incrementado de manera exponencial. Actualmente se reconoce la existencia de 33 especies, las cuales han establecido procesos de coevolución estrechos con múltiples grupos de mamíferos, en especial carnívoros (*B. hanselae*) y roedores (*eg*. *B. grahamii*, *B. elizabethae, B. vinsonii*) (Heller et al. 1997; Kosoy et al. 2012; Lei y Olival, 2014) (Fig. 5).

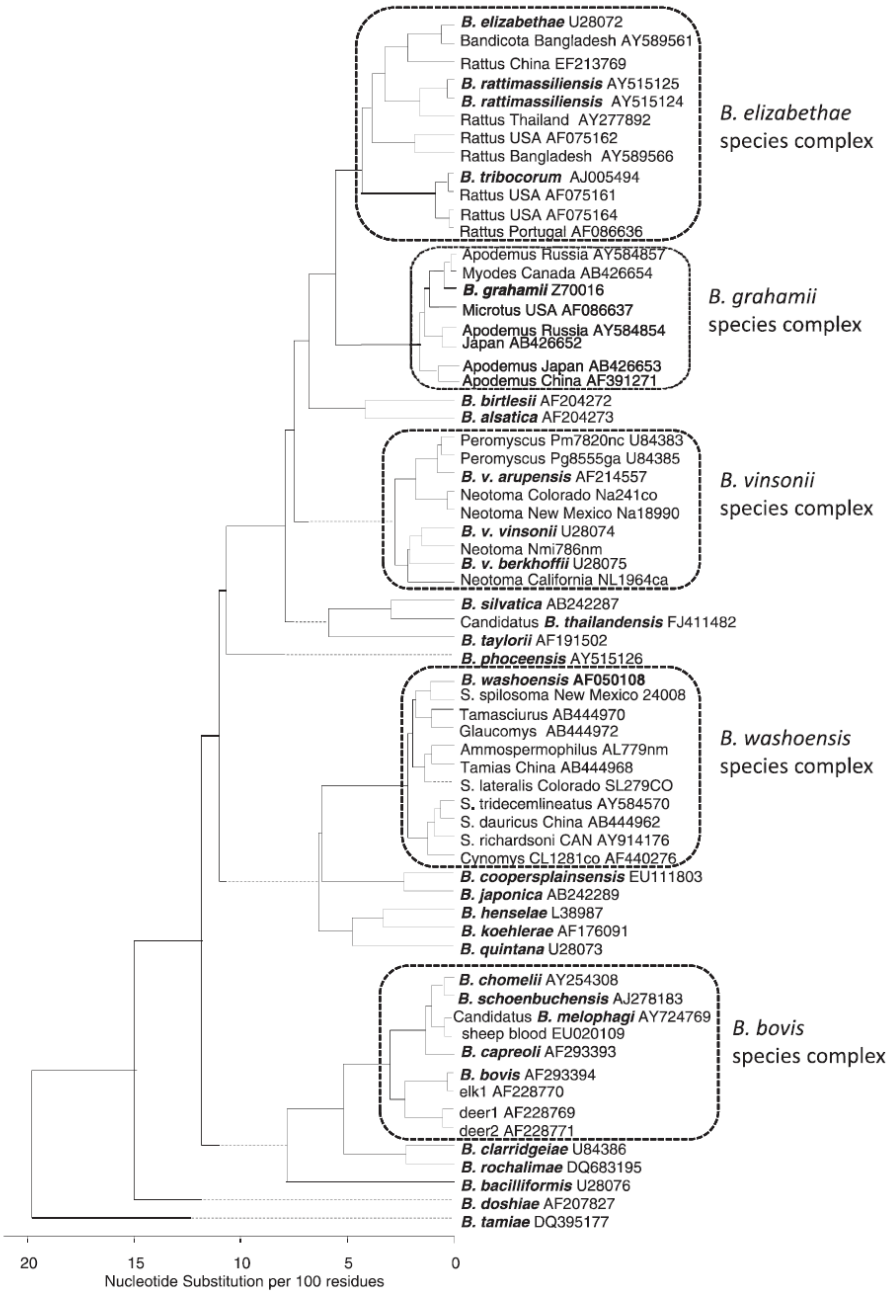

Figura 5. Filogenia de las especies del género *Bartonella* (Tomado de Kosoy et al. 2012)

La trasmisión se presenta por vía vectorial, a través de múltiples taxa de vectores, como moscas hematófagas, piojos y pulgas, los cuales se infectan al momento de alimentarse de un hospedero infectado. Las bacterias viajan a través del tracto digestivo y se reproducen en el intestino, siendo secretadas con las heces durante toda la vida del ectoparásito. Una vez que el hospedero vertebrado entra en contacto con las heces del

ectoparásito, ya sea al momento de rascarse, durante el lamido o por aerosoles, la bacteria entra en la circulación sanguínea y coloniza múltiples líneas celulares (*eg.* células endoteliales, células fagocíticas) durante tres o cuatro días. Durante este periodo adquiere factores de virulencia que le permiten invadir los eritrocitos y mantenerse en un ciclo intraeritrocítrico persistente (bacteremia generalizada) (Dehio 2005; Chomel et al. 2009; Kempf et al. 2010; Harms y Dehio, 2012) (Fig. 6).

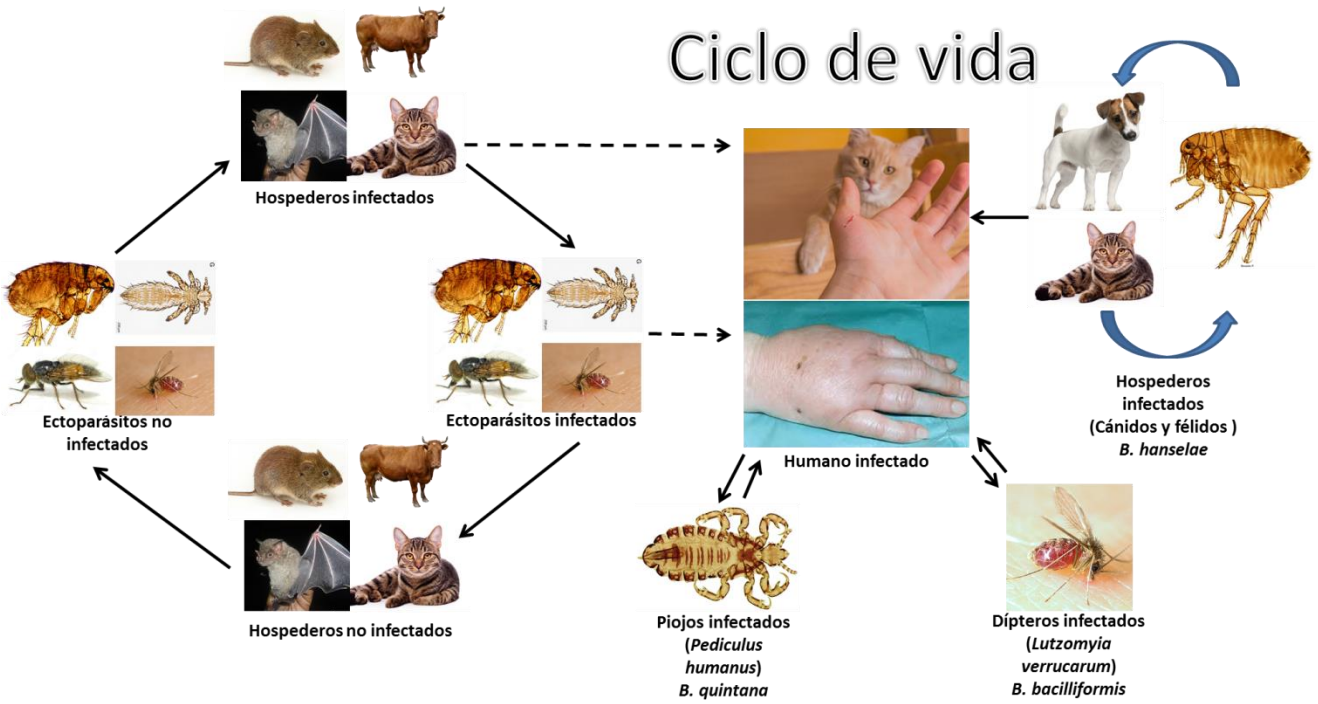

Figura 6. Ciclo de vida de las especies del género *Bartonella*

El ser humano es un hospedero accidental en el ciclo de vida de estas bacterias, con excepción de *B. quintana* y *B. bacilliformis*, agentes de la Fiebre de las Trincheras y la Fiebre de Oroya respectivamente, las cuales tienen como único reservorio vertebrado al ser humano y que constituyen dos padecimientos de suma importancia para la salud pública (Alcántara et al. 2009; Sangaré et al. 2014). La fiebre de las trincheras se transmite por el piojo del cuerpo (*Pediculus humanus humanus*) y se caracteriza por la presencia de un

cuadro febril con presencia de artralgias y mialgias severas al cabo de 15 días del contagio. La fiebre aparece en oleadas cada cinco días y dependiendo del estado de inmunocompetencia del hospedero la infección pude resolverse de manera espontánea o puede presentar una elevada mortalidad. En contraparte la fiebre de Oroya es un padecimiento restringido a Sudamérica, particularmente en Colombia, Perú y Ecuador, el cual es trasmitido por diversas especies de dípteros del género *Lutzomyia*. El patógeno puede generar dos cuadros: uno febril agudo con presencia de anemia hemolítica que puede contribuir a una elevada letalidad de hasta el 85% de los casos y la otra crónica, la cual se conoce como verruga peruana y presenta lesiones cutáneas de tipo papulosa y pruriginosas que pueden persistir durante meses. El tercer padecimiento de importancia en salud humana es la Fiebre por rasguño de gato, producida por *Bartonella hanselae,* en la cual cánidos y félidos son reservorios de la bacteria y la adquieren a través del contacto con heces de pulgas (*Ctenocephalides felis*). El ser humano adquiere la infección por arañazo o mordedura del vertebrado; una vez que la bacteria ha ingresado al sitio de la laceración, transcurren de tres a 20 días del periodo de incubación y posteriormente genera pápulas eritematosas en el sitio de la lesión y linfadenopatía localizada (Heller et al. 1997; Jacomo et al. 2002; Alcántara et al. 2009).

El diagnóstico de estas patologías depende de las especies y la condición clínica del paciente, utilizándose la detección de anticuerpos IgG mediante la implementación de una ELISA. Sin embargo, actualmente se considera que la amplificación de los genes *gltA* y 16S-RNA mediante PCR punto final y tiempo real a partir de muestras de sangre y biopsias de pápulas son los métodos más sensibles que adicionalmente permiten brindar una identificación especie-específica del agente etiológico (La Scola y Raoult ,1999; Chomel et al. 2003; La Scola et al. 2003; Kosoy et al. 2012; Angkasekwinai et al. 2014).

El tratamiento se basa en el suministro de antibióticos con acción bacteriostática, como la azitromicina y la rifampicina, sin embargo, el antibiótico de primera elección es la doxiciclina en cualquiera de los tres padecimientos antes mencionados (Chomel et al. 2003).

Justificación

Las limitaciones en métodos diagnósticos basados en pruebas serológicas tardadas y con baja especificidad han impedido conocer la incidencia real de leptospirosis, rickettsiosis y bartonelosis en México, así como la distribución de vectores y reservorios que determinan su persistencia. La identificación de la dinámica de transmisión en el medio silvestre y peri-doméstico permitirá implementar la Vigilancia Epidemiológica Activa para lograr un mejor control y prevención de estas enfermedades zoonóticas emergentes. Así mismo, el conocer los factores que participan en la transmisión de estas zoonosis, permitirá identificar potenciales zonas de riesgo donde puede ocurrir el contacto entre individuos susceptibles y los patógenos.

Para resolver esta problemática, el presente estudio se enfoca a implementar el diagnóstico molecular específico para la identificación de los tres grupos de patógenos. Con estas pruebas moleculares se estudiará la diversidad y prevalencia de las especies de *Leptospira*, *Rickettsia* y *Bartonella* en poblaciones de mamíferos silvestres y sus ectoparásitos asociados en múltiples focos donde se han reportado casos humanos de estas enfermedades.

Objetivos

Objetivo General

Identificar la prevalencia de *Leptospira* sp., *Rickettsia* sp. y *Bartonella* sp. en poblaciones de roedores silvestres y peridomésticos, y sus ectoparásitos asociados en los estados de Jalisco, Hidalgo y San Luis Potosí.

Objetivos Particulares

1) Identificar el panorama epidemiológico de la leptospirosis, rickettsiosis y bartonelosis humanas en México.

2) Identificar molecularmente las especies de *Leptospira*, *Rickettsia* y *Bartonella* en roedores y ectoparásitos asociados en tres estados de México con presencia de casos de leptospirosis, rickettsiosis y bartonelosis en humanos.

3) Identificar las especies de roedores y ectoparásitos que pueden fungir como reservorios/vectores de las especies de *Leptospira*, *Rickettsia* y *Bartonella* detectados.

4) Determinar la prevalencia de las especies de *Leptospira*, *Rickettsia* y *Bartonella* en las especies de roedores y ectoparásitos recolectadas.

Material y Método

Selección de localidades

Para la selección de localidades de colecta, se realizó una búsqueda intencionada de casos humanos de Bartonelosis, Rickettsiosis y Leptospirosis. Para ello se obtuvieron datos de: **1) casos confirmados** de la base de datos "Anuarios de Morbilidad durante el período 1995-y 2014"; **2) casos probables** provenientes del "Sistema Único de Vigilancia Epidemiológica" (SUIVE), ambos generados por la Secretaría de Salud (CENAVECE, 2014) y, **3) defunciones**, las cuales se obtuvieron de la base de datos "Estadísticas de Mortalidad" del Instituto Nacional de Estadística y Geografía (INEGI, 2014). Se seleccionaron los estados donde la incidencia de casos fue superior a 5 por cada 100,000 habitantes para Leptospira o bien que hayan presentado al menos un registro de Bartonelosis o Rickettsiosis. Adicionalmente para la sub-selección de los municipios, se tomó en cuenta la coexistencia de casos por dos o tres patógenos en el mismo sitio o que fuesen localidades aledañas a donde se han registrado los mismos. Los estados que cumplieron con estas condiciones fueron Hidalgo, Jalisco y San Luis Potosí.

Trabajo de campo

En las localidades de Hidalgo, Jalisco y San Luis Potosí donde se han presentado casos humanos de Bartonelosis, Rickettsiosis y Leptospirosis, se realizó búsqueda intencionada de estos tres patógenos en mamíferos pequeños y ectoparásitos asociados (mediante el permiso de colector científico FAUT-0170 expedido por SEMARNAT). Los roedores se recolectaron utilizando trampas Sherman, en las cuales se colocó un cebo que consistió en una mezcla de avena-vainilla. Se colocaron cuatro transectos de 40 trampas cada uno, durante tres noches consecutivas (Romero-Almaraz et al. 2007). Los ejemplares recuperados se individualizaron en bolsas de plástico para prevenir la contaminación cruzada de ectoparásitos y se sacrificaron cumpliendo las directrices de la Sociedad Americana de Mastozoología para el Uso de Mamíferos Silvestres en Investigación (Gannon y Sikes, 2011). Se realizó la necropsia y se extrajo una porción del hígado, bazo y oreja izquierda, los cuales se preservaron en etanol absoluto. De manera paralela se recuperaron garrapatas, piojos y pulgas mediante extracción manual, a partir de la acuciosa revisión de los mamíferos con ayuda de microscopio estereoscópico, los cuales también se fijaron en etanol absoluto.

Trabajo de laboratorio

Identificación taxonómica de mamíferos

Los ejemplares se identificaron mediante el uso de literatura especializada y se depositaron (piel, cráneo y esqueleto, así como tejidos congelados) en la Colección Mastozoológica del Museo de Zoología "Alfonso L. Herrera" de la Facultad de Ciencias, UNAM.

Identificación morfológica de ectoparásitos

Para la identificación específica piojos y pulgas, estos se montaron en laminillas mediante las técnicas de Kim et al. (1986) y Wirth y Marston (1968), mientras que las garrapatas se mantuvieron en viales con etanol absoluto. Los ejemplares se identificaron mediante el uso de las claves taxonómicas especializadas de Kim et al. (1986) para piojos; Acosta y Morrone (2003), Hastriter (2004), Hopkins y Rothschild (1971), Morrone et al. (2000) y Traub (1950) para pulgas y Guzmán-Cornejo et al. (2011), Nava et al. (2014) y Martins et al. (2010, 2014) para garrapatas. Los ejemplares se depositaron en la Colección de Siphonaptera Alfredo Barrera, del Museo de Zoología "Alfonso L. Herrera" en la Facultad de Ciencias y la Colección del Centro de Medicina Tropical de la Facultad de Medicina, ambas pertenecientes a la Universidad Nacional Autónoma de México.

Extracción de DNA

Se llevó a cabo la extracción de DNA a partir de las muestras de hígado, bazo y oreja en reservorios y de los ectoparásitos asociados, con ayuda del kit DNeasy Blood & Tissue de Qiagen y el protocolo de Purificación de DNA total procedente de tejidos.

Identificación molecular de los ectoparásitos

Para la identificación molecular se amplificó un fragmento de 400 pb del gen Citocromo Oxidasa Subunidad I (COI) (Cuadro 1).

Cuadro 1. Oligonucleótidos utilizados en este estudio.

Organismo	Gen	Primers	Secuencia (5´-3´)	Tamaño (bp)	Referencia
Ectoparásitos	*COI* (Citocromo oxidasa subunidad I)	L6625	CCGGATCCTTYTGRTTYTTYGGNCAYCC	400	Hafner et al. 1994
		H7005	CCGGATCCACNACRTARTANGTRTCRTG		
Rickettsia sp.	*gltA* (Citrato sintasa)	RpCS.415	GCTATTATGCTTGCGGCTGT	806	de Souza et al. (2006)
		RpCS.1220	TGCATTTCTTTCCATTGTGC		
	ompB (Proteína exterior de membrana B)	120-M59	CCGCAGGGTTGGTAACTGC	862	Roux y Raoult, 2000
		120–807	CCTTTTAGATTACCGCCTAA		
	ompA (Proteína exterior de membrana A)	Rr190.70	ATGGCGAATATTTCTCCAAAAA	532	Regnery *et al*. (1991)
		Rr190.602	AGTGCAGCATTCGCTCCCCCT		
Bartonella sp.	*gltA* (Citrato sintasa)	BhCS781.p	GGGGACCAGCTCATGGTGG	379	Norman et al. 1995
		BhCS1137.n	AATGCAAAAAGAACAGTAAACA		
Leptospira sp.	*16S rRNA*	16S-F	GAACTGAGACACGGTCCAT	430	Vital-Brazil et al. 2010
		16S-R	GCCTCAGCGTCAGTTTTAGG		

Detección molecular de patógenos

Se realizó la PCR con el DNA proveniente de las muestras utilizando los iniciadores que son específicos para cada grupo bacteriano (Cuadro 1). La mezcla de reacción incluyó 12.5 μL de GoTaq® Green Master Mix, 2X de Promega Corporation (Madison, WI, USA), 100 ng de cada oligo, 6.5 μL de agua libre de nucleasas y 200 ng de DNA en un volumen final de 25 μL. Se incluyeron un control negativo (Mezcla de reacción con agua libre de nucleasas) y controles positivos para cada grupo de (DNA de *Bartonella henselae*, *Leptospira interrogans*, *Rickettsia rickettsii*). Los productos de PCR se analizaron mediante

electroforesis en geles de agarosa al 1.5%, utilizando el marcador de peso molecular de 100 pb (LMW DNA Ladder de BioLabs) en buffer TAE 1X. Los geles se tiñeron con el fluorocromo SYTO® 60 (Invitrogen, Life Technologies CA, USA) y se visualizaron mediante el uso del fotodocumentador ODYSSEY CLx (LICOR Biosciences).

Secuenciación

Los productos de PCR que resultaron positivos fueron enviados al Laboratorio de Biología Molecular de la Biodiversidad y la Salud del Instituto de Biología de la UNAM donde fueron preparados para ser leídos en un secuenciador Life Technology modelo 3500xl.

Identificación específica de los patógenos detectados y análisis de su posición filogenética

Una vez obtenidas las secuencias, se compararon con las disponibles en GenBank mediante el algoritmo Basic Local Alignment Search Tool [BLAST]. Para identificar las especies de *Bartonella*, *Leptospira* y *Rickettsia* detectadas, se implementaron los criterios de La Scola (2003); Fournier y Raoult (2009); Fournier et al. (2003); Wayne et al. (1987) y Amann et al. (1992). Una vez corroborada la identidad de las secuencias estas se alinearon junto con las de referencia mediante el algoritmo Clustal W, con ayuda del software MEGA 6.0. Paralelamente se seleccionó el modelo de sustitución nucleotídica basado en el valor BIC (Criterio Bayesiano de Información) más bajo y se calcularon las divergencias genéticas, a partir de las cuales se generaron dendogramas o árboles filogenéticos utilizando los métodos de Neighbor-Joining y Máxima Verosimilitud con un soporte de los clados mediante 1,000-10,000 réplicas de Bootstrap respectivamente.

RESULTADOS

Capítulo 1 Leptospirosis in Mexico: Epidemiology and Potential Distribution of Human Cases

PLOS | ONE

RESEARCH ARTICLE

Leptospirosis in Mexico: Epidemiology and Potential Distribution of Human Cases

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Abstract

Background

Leptospirosis is widespread in Mexico, yet the potential distribution and risk of the disease remain unknown.

Methodology/Principal Findings

We analysed morbidity and mortality according to age and gender based on three sources of data reported by the Ministry of Health and the National Institute of Geography and Statics of Mexico, for the decade 2000–2010. A total of 1,547 cases were reported in 27 states, the majority of which were registered during the rainy season, and the most affected age group was 25–44 years old. Although leptospirosis has been reported as an occupational disease of males, analysis of morbidity in Mexico showed no male preference. A total number of 198 deaths were registered in 21 states, mainly in urban settings. Mortality was higher in males (61.1%) as compared to females (38.9%), and the case fatality ratio was also increased in males. The overall case fatality ratio in Mexico was elevated (12.8%), as compared to other countries. We additionally determined the potential disease distribution by examining the spatial epidemiology combined with spatial modeling using ecological niche modeling techniques. We identified regions where leptospirosis could be present and created a potential distribution map using bioclimatic variables derived from temperature and precipitation. Our data show that the distribution of the cases was more related to temperature (75%) than to precipitation variables. Ecological niche modeling showed predictive areas that were widely distributed in central and southern Mexico, excluding areas characterized by extreme climates.

Conclusions/Significance

In conclusion, an epidemiological surveillance of leptospirosis is recommended in Mexico, since 55.7% of the country has environmental conditions fulfilling the criteria that favor the presence of the disease.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Leptospirosis is a zoonotic disease considered one of the most widely disseminated and prevalent in the wild, which can be transmitted by direct or indirect contact with urine of infected animals [1–5]. Humans are accidental hosts in the natural life cycle of the bacteria, and can show a wide range of symptoms such as febrile illness, headache, prostration, severe myalgia, uveitis (some of which may be mistaken with other infectious diseases like dengue, flu, hantavirus and rickettsiosis) [3], even renal failure and haemorrhagic manifestations also known as Weil's disease. However, some infected people never show symptoms, having only a subclinical infection [1,2,4].

In Mexico, the first report of leptospirosis was made in patients from Merida, Yucatan, which had originally been diagnosed with yellow fever, yet the isolation of the bacteria proved leptospirosis [6]. Thereafter, a number of papers published between 1920 and 1990 reported the prevalence of the disease mostly in the southeast part of the country [7–10], yet little is known of the prevalence in the entire nation [11–13]. A report on the annual incidence of leptospirosis (one case per million inhabitants) done in 2007 showed that the majority of cases focalized in the state of Veracruz [14]. A more recent epidemiological overview of leptospirosis in Mexico between 2000–2010 showed a more widespread disease distribution [15].

Leptospirosis has been included in the group of re-emerging infectious diseases [16] and neglected zoonoses [17]. Annually, an estimated of 300,000–500,000 new acute cases emerge worldwide [18], with outbreaks occurring in several Latin-American countries, including Mexico. This calls for an analysis of epidemiological data through spatial epidemiology, which permits a description and analysis of geographical index health data with regard to demographic and environmental risk factors [19]. Epidemiological information can be accompanied by other techniques, such as ecological niche modeling. The combination of both sources of information can bring new perspectives to the analysis of infectious diseases [20–25], given that it is possible to characterize the potential geographic distribution based on environmental parameters [22, 23, 26].

Since leptospirosis remains a neglected disease and only scarce information exists on its impact on public health, our main objective was to identify the regions where leptospirosis could be present in Mexico, based on epidemiological data reported during the period 2000–2010, and also based on the same data, create a potential distribution map to identify the geographic distribution of the environmental variables associated to the presence of the disease. The combination of both sources of information permits the identification of geographical risk areas for the disease in Mexico and aids the design of preventive actions to limit the spread of the disease.

Materials and Methods

Epidemiological Data

We analysed morbidity and mortality data of leptospirosis in Mexico during the decade 2000–2010. The present analysis was done with three sources of data published by the Health Ministry and the National Institute of Geography and Statics of Mexico, based on the guidelines of *Norma Oficial Mexicana PROY-NOM-017-SSA2-2012 (para la vigilancia epidemiológica que establece la obligatoriedad y procedimientos generales de vigilancia de casos de Leptospirosis).* The data do not include names or personal identification data of individual patients. This study was approved by the Ethics and Research Committee of the Medical Faculty of the UNAM (Universidad Nacional Autónoma de México) (FMED/CI/JMO/004/2012). Incidence data were divided into confirmed cases (patients that presented suggestive symptomatology of

the disease and antibody titers of 1:800, including confirmation with a second sample in which the titers increased four times the initial value) and probable cases (patients with suggestive symptomatology of the disease and positive leptospira microagglutination or ELISA tests), according to the "Mexican Official Norm NOM-029-SSA2-1999, for the epidemiological surveillance, prevention and control of Leptospirosis in humans" [Norma Oficial Mexicana NOM-029-SSA2-1999, para la vigilancia epidemiológica, prevención y control de la Leptospirosis en el humano] [27]. We obtained data of: 1) confirmed cases from the database "Morbidity Yearbook during the period 1995–2010" [Anuarios de Morbilidad durante el periodo 1995–2010] and 2) for probable cases from the "Unique Information System for Epidemiological Surveillance [Sistema Único de Vigilancia Epidemiológica] (SUIVE)", both generated by Ministry of Health of Mexico [Secretaría de Salud] [28]. The last source, 3) included data on deceased leptospirosis cases that were obtained from the database "Mortality Statistics" [Estadísticas de Mortalidad] of the National Institute of Geography and Statics of Mexico [Instituto Nacional de Estadística y Geografía] [29], that grouped the cases according to the International Statistical Classification of Diseases and Related Health Problems (ICD-10) [30]. Additionally, population statistics were obtained from the database "Basic Demographic Indicators 1990–2030" [Indicadores Demográficos Básicos 1990–2030] of the same institute [29].

It is important to clarify that data on specific serovars were not available for this study.

Analysis of cases was conducted according to age and gender of the patients and according to administrative entities (state) that notified the case by applying the Student's t-test for comparison of genders and the Kruskal-Wallis test (to compare mean values of patient ages and the state where patients had been reported) with post-hoc pairwise comparisons using Mann-Whitney U tests with Bonferroni correction. All tests were two tailed and p values <0.05 were considered significant. All statistical analyses were conducted using SPSS 20 [31, 32]. We obtained the annual incidence rates with the middle year population based on data from National Population Council of Mexico. We standardized rates with population of Mexico from 2005 with the use of Epidemiological Software Epidat 3.1 [33].

For mortality analysis, we conducted an analysis of specific mortality rates and case fatality ratio. In both cases the same multivariate analysis that had been used for morbidity data was included.

Potential Distribution Map

Disease records. We obtained the geographic locations where the disease had been reported. Our records were divided in two groups defined as: validated cases [obtained from administrative entities that notified confirmed cases and deaths], and non-validated cases [including probable cases and deaths that were pooled together due to the unknown status of the patients and the possibility that they had not been infected in the administrative entity] [29]. The georeferentiation was based on data from "Catalogue of Keys from the Federal States, Municipalities and Localities" [Catálogo Único de Claves de Áreas Geoestadísticas Estatales, Municipales y Localidades] [34].

Ecological niche modeling approach and potential distribution. To create the potential distribution of the disease, we used a dataset of 19 bioclimatic variables, derived from temperature and precipitation, as shown in Table 1. Values of the bioclimatic variables were calculated based on the means of climatic records from 1910 to 2009 specifically for Mexico [35].

These layers correspond to climatic records from 1910 to 2009, which were specifically recovered for Mexico with a grid cell size of 0.01° x 0.01° (approx. 1 km2 x 1 km2) [35]. Although the inclusion of all 19 bioclimatic variables is prone to over-fitting [36], their use

Table 1. Bioclimatic variables derived from temperature and precipitation.

Bio1	Annual mean temperature
Bio2	Mean diurnal range (mean monthly min. temp.—mean monthly max. temp.)
Bio3	Isothermality (Bio2/Bio7)
Bio4	Temperature seasonality (SD)
Bio5	Maximum temperature of the warmest month
Bio6	Minimum temperature of the coldest month
Bio7	Annual temperature range (Bio5-Bio6)
Bio8	Mean temperature of the wettest quarter
Bio9	Mean temperature of the driest quarter
Bio10	Mean temperature of the warmest quarter
Bio11	Mean temperature of the coldest quarter
Bio12	Annual precipitation
Bio13	Precipitation of the wettest month
Bio14	Precipitation of the driest month
Bio15	Precipitation seasonality (coefficient of variation)
Bio16	Precipitation of the wettest quarter
Bio17	Precipitation of the driest quarter
Bio18	Precipitation of warmest quarter
Bio19	Precipitation of coldest quarter

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represents a conservative and a more reliable approach to estimate the potential distribution of the disease [37].

The potential distribution models were created using the Genetic Algorithm for Rule-set Production (GARP) implemented in the Desktop GARP software [38], which has proved to be a useful tool in understanding geographic and ecological distributions. GARP works in an iterative process through the formation of rules using inferential characteristics (atomic, range, negated range and logistic regression), the rules are evaluated and then considered to pass, or not, to a next generation. The process is stopped when a limited number of iterations are reached or when there is a limit of convergence and the new rules do not improve the model [39, 40].

A total of 100 replicates were generated using only the validated cases (those with information of prevalence and/or icterohemorrhagic leptospirosis). The data were split into training and testing points in a proportion of 70%–30%, respectively, in random seed to assure the randomness of the data to generate each replicate. Each replicate was made considering a limit of convergence of 0.01, which means that rules could not improve the result of the model in more than 1%, or when 1000 iterations were reached. The replica were validated by using a χ^2 test, which considers the training and testing data, to evaluate the predictive capacities in each replicate used for binary predictions [41–43]. Then, from the 100 replicates, we selected the 10 best models based on minor omission errors and commission values close to the median [44]. These distribution models were summed to obtain only one final consensus model. This model was used to establish two thresholds of presence/absence of the disease, one for 90% of the best subset models [43] and other with 100% of the models.

Given that the non-validated data are not informative for epidemiological analyses, we decided to evaluate if it was possible to predict them using our final model. In order to prove that the association of the non-validated data was not the same as expected by chance, we used 100 sets of random points, where each set was formed with the same number of non-confirmed data.

To identify the values of the variables that could explain the potential distribution of the disease, we analysed the final model and the bioclimatic variables through a recursive partitioning analysis and a classification and regression tree, which are statistical procedures based on non-parametric regression methods [45]. Due to the large amount of pixels to be analysed in the final model, we resampled the geographical extent using a regular net of points separated every 5 km that represent a regular subsample of the presence/absence data in the model. The values of the bioclimatic layers were associated for each point in the net, and the classification and regression tree was constructed using JMP 9 [46]. The values considered on the tree were those present in the potential distribution model and that show a cumulative probability, based on the values of the variables considered in a nested form [45, 46].

The creation of the map was using the geographic information system QGIS 2.8.2-Wien (GNU General Public License) based on our presence point data and the results of the ecological niche modeling. We used the administrative areas of the World from the Global Administrative Areas 2.0 [47] and the Mexican administrative areas were provided by CONABIO [48] under licence CC BY-NC 2.5 MX (http://creativecommons.org/licenses/by-nc/2.5/mx).

Results

Epidemiological Overview

Distribution of cases and incidence. During the decade of 2000–2010, 1,547 confirmed cases were registered in 89 municipalities of 27 (84.4%) of the 32 states in Mexico. The state with highest number of cases was Veracruz with 377 (24.4%), followed by Tabasco 260 (16.8%), Sinaloa 129 (8.3%), Hidalgo 116 (7.5%) and Oaxaca 97 (6.3%). The states with lowest number of cases were Nayarit and Tlaxcala 3 (0.2%), Michoacan 2 (0.1%) and Aguascalientes 1 (0.1%). States that never reported cases were: Baja California, Durango, Guanajuato, Queretaro and Zacatecas (Table 2).

A significant difference is observed when comparing the mean annual data of cases obtained in the states: Distrito Federal, Hidalgo and Veracruz ($\chi^2 = 35.88$, $p = <0.001$).

The number of cases by year ranged from 40 to 483, with a median of 141 annual cases. Incidence rate varied from (0.04) to (0.44) per 100,000 inhabitants. During the entire period, the number of cases increased 12 times, whereas the incidence rate rose 11 times.

We observed two peaks: one occurred in 2007 with 223 cases (an incidence rate of 0.21 per 100,000 inhabitants) and one in 2010 with 483 cases (incidence rate of 0.44 per 100,000 inhabitants) (Fig 1).

During the whole period 2000–2010, the majority of cases were reported during the rainy season (throughout the second semester of the year). A total of 1,121 cases (72.5%) were reported during July through December, with the increase in the registration beginning in June (with a mean number of 60 to 80 cases), reaching the highest number in October (with 279 cases), which represents an increase of 3.4 times, as compared to previous months (Fig 2).

Age and gender. We could not analyse the incidence according to gender for the entire period since the Mexican Ministry of Health began notifying the gender of the patients after 2002. However, the analysis done from 2003–2010 showed no significant differences between male and female cases, where 760 (55.4%) cases were male and 613 (44.6%) were female, and incidence rates between both groups showed no significant differences (Table 3).

The age group showing the highest number of cases was 25–44 years with 554 cases (35.8%), followed by the age group of 15–19 with 182 cases (11.8%). The lowest number of cases corresponded to children aged less than one year, with only 4 cases. With regard to infection rates, the 50–59 year age group had the highest infection rate (1.72 per 100,000

Table 2. Epidemiological overview of leptospirosis by states in Mexico, during the period 2000–2010.

State	Cases	Deaths	Incidence rate*	Specific mortality rate*	Case fatality ratio (%)
Aguascalientes	1	0	0.08	0	0
Baja California	0	0	0	0	0
Baja California Sur	5	0	0.8	0	0
Campeche	23	6	2.82	209.57	26.09
Coahuila	4	0	2.77	0	0
Colima	6	0	0.62	0	0
Chiapas	75	2	0.08	11.75	50
Chihuahua	4	1	0.18	5.78	16.67
Distrito Federal	80	44	0.92	70.82	55
Durango	0	0	0	0	0
Guanajuato	0	1	0	4.31	0
Guerrero	65	9	1.93	79.93	13.85
Hidalgo	116	0	4.39	0	0
Jalisco	10	2	0.14	5.76	20
Mexico State	47	6	0.31	12.32	12.77
Michoacan	2	1	0.05	5.03	50
Morelos	5	2	0.29	25.51	40
Nayarit	3	0	0.28	0	0
Nuevo Leon	4	1	0.09	5.02	25
Oaxaca	97	3	2.62	10.85	2.02
Puebla	16	3	0.28	7.11	12.5
Queretaro	0	0	0	0	0
Quintana Roo	16	2	1.22	32.78	6.25
San Luis Potosi	35	0	1.37	0	0
Sinaloa	129	65	4.68	585.31	51.94
Sonora	92	22	3.48	166.20	22.83
Tabasco	260	11	11.74	121.55	4.23
Tamaulipas	16	1	0.5	7.16	6.25
Tlaxcala	3	1	0.26	22.18	33.33
Veracruz	377	14	4.99	37.67	3.71
Yucatan	56	2	2.88	31.22	5.36
Zacatecas	0	0	0	0	0
Total	1547	198	-	-	-

*Rate per 100,000 inhabitants

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inhabitants) as compared to children younger than one year of age, showing 0.20 per 100,000 inhabitants (Fig 3).

Specific Mortality and Case Fatality Ratio. A total of 198 confirmed deaths were registered in 21 states of Mexico during 2000–2010 (Table 2). The number of deaths differed significantly between the states ($\chi^2 = 112.304$, $p = <0.001$). States with high number of deaths include Sinaloa with 65, followed by Distrito Federal with 44. In contrast, Chihuahua, Guanajuato, Michoacan, Nuevo Leon, Tamaulipas and Tlaxcala notified only one case during the notification period. Interestingly, although Hidalgo exhibited a high incidence rate (4.39 per 100,000), this state reported no deaths (Table 2).

Throughout the decade, the specific mortality rate for leptospirosis varied between 1.25 and 5.86 per 100,000, which triplicated during 2004–2006. The case fatality ratio during this decade

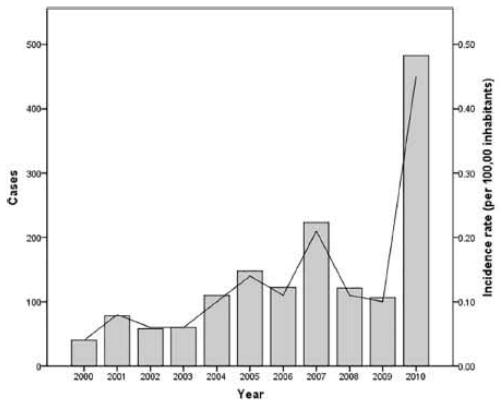

Fig 1. Distribution of cases and incidence rate of leptospirosis registered in Mexico during the period 2000–2010. Number of cases are represented by bars and the incidence rate is represented by the solid line.

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exhibited a mean value of 12.8% (198/1547), where the highest ratio was registered in 2000 with 33% (13/40). Although the ratio decreased to 11.4% during the following five years, a renewed temporal increase surged in 2006 showing a 24% (29/122) case fatality ratio, which diminished drastically in 2010 to 5% (23/483), reaching the lowest value of the decade (Fig 4).

We additionally obtained information on the type of locality (rural/urban) where the 198 deaths were reported. Significantly higher numbers of deaths were reported in urban areas with 161 cases (81.3% with a mean of 14.5 deaths per year) against 35 (17.7% with a mean of 3.5 deaths per year) reported in rural settings, $p = 0.0005$; only 2 cases (1.0%) were classified as unknown.

The analysis of the deceased patients according to gender, revealed higher numbers (121) in male (61.1%) in contrast to 77 female patients (38.9%), albeit the difference was not significant

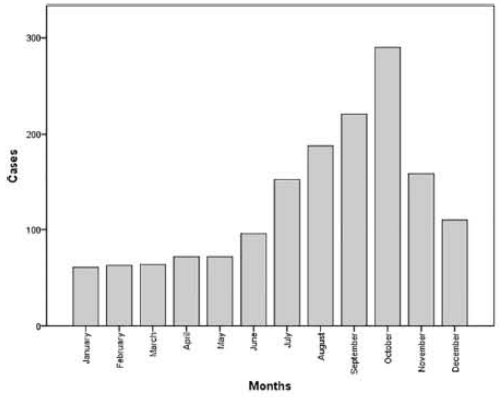

Fig 2. Monthly distribution of leptospirosis cases in Mexico during the period 2000–2010. Number of cases are represented by bars.

doi:10.1371/journal.pone.0133720.g002

Table 3. Cases and incidence rate of leptospirosis according to gender, during the period 2003–2010 in Mexico.

	General		Females			Males		
Year	Cases	%	Cases	%	Incidence rate*	Cases	%	Incidence rate*
2003	60	4.4	24	3.9	0.07	36	4.7	0.05
2004	110	8.0	61	10	0.1	49	6.4	0.12
2005	148	10.8	67	10.9	0.15	81	10.7	0.13
2006	122	8.9	52	8.5	0.13	70	9.2	0.1
2007	223	16.2	92	15.0	0.25	131	17.2	0.17
2008	121	8.8	54	8.8	0.13	67	8.8	0.1
2009	106	7.7	45	7.3	0.11	61	8.0	0.1
2010	483	35.2	218	35.6	0.50	265	34.9	0.39
Total	1373	100	613	100		760	100	

*Rates per 100,000 inhabitants. $\chi^2 = 112.304$, $p = >0.005$

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($p = 0.105$). A significant difference in case fatality ratios was observed between genders, being 17.5% (107/613) for males and 8.4% (64/760) for female patients ($p = 0.034$).

There was a significant difference in the number of deaths from leptospirosis according to age ($\chi^2 = 45.911$, $p = <0.001$). The age group of 25–44 years showed 47 deaths (24%), followed by adults over age 65 with 36 deaths (18%) and adults 50–59 with 29 fatalities (15%). No deaths were recorded in children with less than one year of age, which differs significantly from the other age groups that report high numbers. The mortality rate increases with age, with the highest value recorded in the larger group of >65, followed by 60–64 years. Although the number of deaths is concentrated in the group of 25–44 years, the mortality rate decreases because it comprises a much wider group of people (Fig 5).

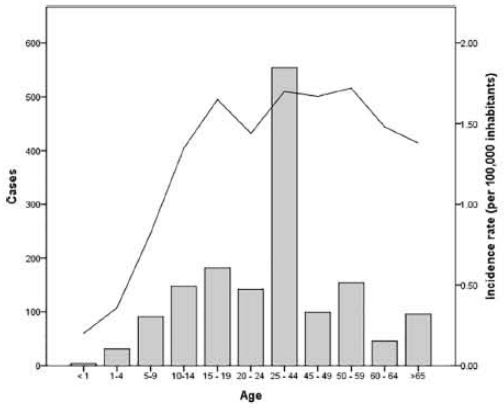

Fig 3. Cases and infection rates according to age. The bars represent the number of cases for each age group that was registered during the decade. The solid line represents the incidence rate per 100,000 inhabitants).

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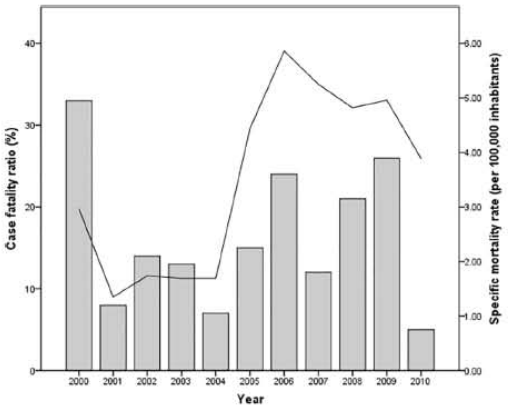

Fig 4. Distribution of leptospirosis case fatality ratio and specific mortality rate registered in Mexico during 2000–2010. The bars represent case fatality ratios (%) and solid line represents specific mortality rates (per 100,000 inhabitants) registered during the decade.

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Ecological Overview

Ecological niche modeling and potential distribution. We obtained a database with 88 spatially unique validated records, and 123 spatially unique non-validated data. Potential distribution was based on the validated data, showing wide predictive areas in the country, mainly in southern Mexico. There was no distribution in regions with extreme climates (extremely cold and dry) and no prediction was evidenced in highlands or in the north of Mexico (Fig 6).

Given the possibility of errors in the geo-references, we took two thresholds into account: the congruence of all best subset models that predicted all validated data (light and dark grey) and a threshold of 96.59% of the best subsets (only the dark grey area), which left out 3.41% of

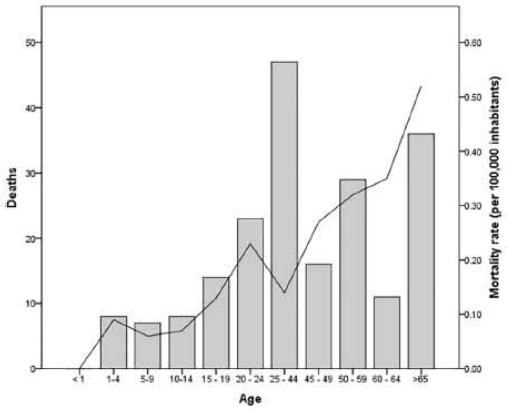

Fig 5. Specific mortality rates and case fatality ratio of leptospirosis according to age. The bars represent number of patient deaths and solid line represents specific mortality rates (per 100,000 inhabitants) registered during the decade.

doi:10.1371/journal.pone.0133720.g005

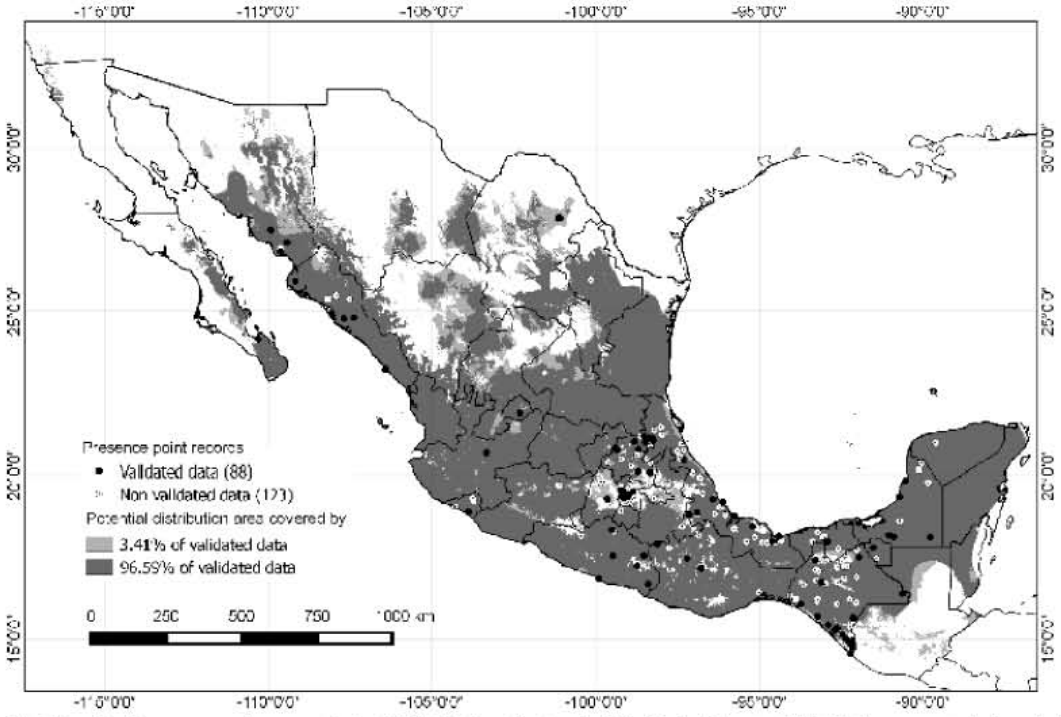

Fig 6. Map showing presence of cases and potential distribution of leptospirosis in Mexico. The potential distribution was created with ecological niche modeling using validated cases. Dark points show localities of validated data (confirmed leptospirosis cases and deaths), light points show non-validated data (probable cases and deaths that were pooled together due to the unknown status of the patients). Grey tones show the two thresholds used to determine the potential distribution of the disease. Dark grey shows the threshold of 96.59% of the best subsets. Light grey shows the remaining 3.41% of the validated data considered as possible errors.

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the validated data. The use of both thresholds showed the possible restrictiveness, if those records that did not coincide with all the best subsets were considered as errors (which corresponded to only 3.41% of the validated data). The restrictiveness is evidenced only in the north and in the highlands of central Mexico, and does not change the pattern previously described (Fig 6).

Despite the fact that non-validated data are not epidemiologically informative, we nonetheless used this model to test if leptospirosis cases could be predicted. The potential distribution map could help clarify if non-validated data could be compared with validated data. The wide potential distribution easily showed that non-validated data were correctly predicted.

The recursive partitioning analysis and the classification and regression tree were calculated for the predictive distribution with the threshold of 100%, which considers the area where all the best subset models coincide. Given the nested characteristics of the analyses, it was important to consider the order of the variables, since the first variables represent a prerequisite for the next variables. It was possible to identify that temperature variables were those that first explained the predictive distribution model, and were also the most informative. The first two temperature variables (Bio4 and Bio2, Table 4) explained up to 75% of the potential

Table 4. Probabilities of presence for leptospirosis in Mexico according to the recursive partition analysis of bioclimatic variables and values.

Cumulative environmental variables		Cumulative probability	Contribution (%)
Bio4 Temperature seasonality (SD)	< 2.009632	0.6195	61.95
Bio2 Mean diurnal range (mean monthly min. temp.—mean monthly max. temp.)	< 16.84381	0.7504	13.09
Bio8 Mean temperature of the wettest quarter	≥ 15.60133	0.8115	6.11
Bio3 Isothermality (Bio2/Bio7)	≥ 0.4397839	0.8498	3.83
Bio3 Isothermality (Bio2/Bio7)	< 0.7535372	0.8811	3.13
Bio13 Precipitation of the wettest month	≥ 15.51667	0.9359	5.48
Bio11 Mean temperature of the coldest quarter	≥ 9.972473	0.9638	2.79
Bio2 Mean diurnal range (mean monthly min. temp.—mean monthly max. temp.)	≥ 9.190779	0.9774	1.36
Bio1 Annual mean temperature	< 27.42323	0.9833	0.59
Bio14 Precipitation of the driest month	< 18.87975	0.9875	0.42
Bio9 Mean temperature of the driest quarter	≥ 11.79974	0.9905	0.3
Bio18 Precipitation of warmest quarter	≥ 87.88699	09922	0.17

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distribution. Precipitation variables, on the other hand, contributed once the temperature variables were already present and their contribution was lower (Table 4).

Discussion

Here we show the first database compilation on leptospirosis in Mexico with an analysis of morbidity and mortality, obtained from three official sources [28, 29]. Since this disease is not officially notified and no official standards for notification exist, the actual numbers of patients remain unknown and the serovars of the *Leptospira* are not always reported in some of the states. Thus, the actual magnitude of the potential distribution and risk of the disease remain unknown. In an attempt to determine the potential distribution of the disease in Mexico, we considered all patients with leptospirosis as a group, irrespective of serovars. When we initiated this analysis, and based on previously published papers on leptospirosis as a water borne disease [1, 2, 4], we expected to find a close correlation between patients with leptospirosis and water. This might hold true for the outbreaks, but precise data of a possible more widespread distribution of the disease throughout the country and factors associated with the disease occurrence have not been analysed. We were interested in studying leptospirosis at a national level, seeking more detailed correlations between the disease distribution with bioclimatic variables derived from temperature and precipitation, in order to clarify which factors play a role in the establishment of the disease.

Leptospirosis had been previously reported as an occupational disease of males [49–51]. Yet our study shows that in Mexico there is no male preference in morbidity, although the case fatality ratio in male patients is significantly higher than in females, which is in accordance with a previous study in Portugal [52]. The overall case fatality ratio reported in Mexico (12.8%) is higher as compared to other countries such as Barbados 9.8%, [53], Morocco 9.5% [54], Germany 8% [55], Trinidad and Tobago 5.8% [56] and Hawaii, USA 0.5% [57]. This is possibly related to problems in the early diagnosis and opportune treatment, yet the underlying cause of the higher male fatality ratio needs to be investigated.

Our data additionally show that disease morbidity has increased during the last decade. This is possibly due to two reasons: in 2010 an active search for patients with dengue was established after several environmental contingencies had occurred (in this period there were a total of 33 tropical cyclones and two hurricanes Agatha and Igor) in the coastal areas of the Pacific and the Atlantic [58, 59]. The second reason for the heightened number of cases is possibly due

to the fact that 17 state laboratories were established (certified for the diagnosis of leptospirosis) and assigned to the National Network of Public Health Laboratories [60], which increased the number of analysed samples. The reduction of patients deaths observed during 2010 is possibly related to a better and opportune early diagnosis enabling a timely treatment.

Within Mexico, the incidence rate and case fatality ratios reported in the different states vary importantly. Furthermore, not all the states that have a high incidence rate also report a large number of patient deaths. This is the case of Hidalgo, a state with the highest incidence rate, yet no patient deaths are reported (Table 2). This could be due to different serovars of the bacteria, which can differ widely in their virulence [2–4]. As most states do not report serovars, this finding calls for the urgent need to report the infecting ones in the affected areas. In addition to serovars, local diagnostic and management facilities must also be taken into account.

Our study also shows that leptospirosis related deaths are predominantly reported in urban areas. This contrasts with the rural distribution of disease morbidity [2, 3]. The cause of this apparent discrepancy can be due to the fact that severely ill patients are transferred to hospitals in major cities, where their decease is finally reported after an obligatory autopsy.

This finding calls for sanitary precautions that need to be attended by health care professionals to avoid infection and undesired spread of the disease.

Our current data open a new perspective of leptospirosis in the country, since it shows a wide scope of environmental factors associated with the disease distribution. Previous reports have only shown pinpoint cases [12], yet our map shows a geographic continuity making it plausible that the disease can have a much wider distribution than expected by sanitary authorities, using models generated for another infectious disease like Chagas disease [61, 62], Dengue [63] and Hantavirus [64]. The analysis of potential distribution based on ecological niche modeling, allows us to associate ecological and biogeographical factors of the pathogen [42, 65]. Even though other biotic factors can be involved in the dynamics of the leptospirosis, such as vectors or host species, these are not easy to represent in the potential distribution hypothesis, because the lack of information does not permit a precise assessment of their specific role, especially if we consider that new host species for leptospirosis are currently being reported in Mexico [66].

Furthermore, our analysis shows that non-validated data share the same ecological characteristics as validated data, and therefore these areas should be followed up closely since they are potentially equally exposed to the disease occurrence. A potential impact of our map is showing that states such as Durango, Queretaro and Zacatecas, that have no epidemiological report of patients, lie within a region likely of having leptospirosis. Thus, it could be possible that the disease is present and should be sought for in humans.

Our study is in accordance with the literature showing that the disease occurs mainly during the rainy season and that it affects predominantly adults [51, 67, 68]. Yet our bioclimatic analysis additionally shows that temperature plays a more determining role in the distribution of leptospirosis cases, as compared to precipitation variables. This finding helps explain the cases reported in desert areas, such as those reported in Israel [69]. Yet this does not hold true for leptospirosis outbreaks, since these are clearly associated with natural disasters such as floods and hurricanes. In Europe, the reported outbreaks were also associated with temperature, since all cases occurred during the hottest summer months [52, 70, 71].

An interesting phenomenon on geographical interference of disease distribution was evidenced in the Distrito Federal, where a high number of cases have been reported (Table 2). A mountain range passes through the southern part of the city forming a corridor that could be responsible for limiting the spread of the disease towards the southern part, which is confirmed by the lack of patients in this geographical region. This contrasts with the northern semi-desert

areas where ecological conditions are permissive for leptospirosis and where patients have been reported.

In conclusion, our findings show that not only precipitation but also temperatures are predictors of the disease distribution. Based on our current analysis, an active epidemiological surveillance of leptospirosis is recommended in Mexico, since 55.7% of the country has ecological conditions fulfilling the criteria to harbour the disease. Clearly, leptospirosis is an emerging disease worldwide that can become a considerable public health problem in Mexico if left unattended.

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Author Contributions

Conceived and designed the experiments: SS DM CR MB IB. Performed the experiments: SS DM CR MB IB. Analyzed the data: SS DM CR MB IB. Contributed reagents/materials/analysis tools: SS DM CR MB IB. Wrote the paper: SS DM CR MB IB.

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Capítulo 2 Panorama epidemiológico de las Rickettsiosis en México durante el periodo 2000-2013

PANORAMA EPIDEMIOLÓGICO DE LAS RICKETTSIOSIS EN MÉXICO DURANTE EL PERIODO 2000-2013

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Resumen

Las Rickettsiosis son enfermedades zoonóticas emergentes causadas por múltiples especies de bacterias intracelulares del género *Rickettsia*, las cuales son transmitidas por diferentes artrópodos vectores (*eg*. garrapatas, piojos, pulgas) y pueden agruparse dentro de los grupos causantes de Tifo y de Fiebres Manchadas.

Debido a que las manifestaciones clínicas son diversas e inespecíficas y las técnicas convencionales de diagnóstico serológico son limitadas para la identificación específica de los agentes etiológicos, el diagnóstico diferencial resulta complejo. Por tal motivo el objetivo del presente trabajo fue identificar el panorama epidemiológico de las principales Rickettsiosis registradas en México durante el periodo 2000-2013. Para ello se realizó un estudio retrospectivo y descriptivo de los casos de Rickettsiosis registrados en el Sistema Único de Vigilancia Epidemiológica (SUIVE) de la Secretaría de Salud.

Se identificó la presencia de 1,297 casos de Rickettsiosis, registrados en 31 entidades federativas, concentrándose la mayoría en los estados de Sonora 316 (24.4%), Sinaloa 265 (20.4%) y Guerrero 155 (12%), careciendo de registros Morelos.

La mayoría se registraron en 2013 con 196 casos (15.1%), en contraste en el 2000 únicamente se registraron 27 (2.1%). La edad de los pacientes osciló en un rango <1-97 años, con un promedio de 24 años. Por otro lado 741 (57%) de los casos se presentaron en mujeres y 556 (43%) en hombres, con una estancia intrahospitalaria de 5 días en promedio.

En cuanto a la clasificación de los casos, 91% de los casos se registraron como Rickettsiosis no especificada y únicamente en 110 de ellos (8.5%) pudo identificarse al agente etiológico (5 *R. akari*, 8 *R. prowazekii*, 80 *R. rickettsii* y 17 *R. typhi*).

Con base en este análisis, resulta de vital importancia la vigilancia epidemiológica activa de las Rickettsiosis en México, ya que se han reportado casos por diversos agentes etiológicos a lo largo de todo el país.

Introducción

Las Rickettsiosis son un conjunto de enfermedades zoonóticas emergentes causadas por bacterias intracelulares obligadas del género *Rickettsia*, las cuales son transmitidas por múltiples artrópodos hematófagos (*eg*. ácaros, garrapatas, piojos, pulgas). En los últimos 20 años con ayuda de técnicas de biología molecular, se ha incrementado de manera exponencial el número de agentes etiológicos que ocasionan estos padecimientos, reconociéndose actualmente la presencia de 26 especies a nivel mundial (Azad y Beard, 1998; Billings et al. 1998; Merhej et al. 2014).

Las Rickettsiosis se clasifican de manera clínica dentro de dos grandes grupos: las Fiebres Manchadas [transmitidas por garrapatas] y los Tifos (endémico o murino y epidémico) [piojos y pulgas], los cuales se manifiestan con síntomas gripales no específicos que incluyen cefalea intensa, congestión conjuntival, fiebre, náusea, rash centrípeto o centrífugo, vómito y dolor abdominal, por lo cual se agrupan dentro del denominado Síndrome Febril Hemorrágico Agudo [SFHA]. También se han reportado signos de meningitis aséptica con confusión mental y rigidez del cuello. Esta fase generalmente dura 4 a 9 días. Entre un 5 y 30% de los casos, los pacientes pueden presentar una forma ictérica severa con presencia de vasculitis, que llevan a falla orgánica múltiple, especialmente hepática, respiratoria, renal y nerviosa (Azad y Beard, 1998; Faccini-Martínez et al. 2014).

El diagnóstico se realiza mediante Inmunofluorescencia indirecta (IFI), sin embargo, esta técnica no es aplicable durante la fase aguda del padecimiento (únicamente se pueden implementar después de los primeros siete días, tiempo necesario para que el paciente haya desarrollado anticuerpos) y presenta elevada reactividad cruzada entre las especies de *Rickettsia*, lo cual dificulta el establecimiento de un tratamiento temprano y oportuno con doxiciclina, por lo que el pronóstico de la mayoría de los pacientes es poco alentador. La mortalidad puede ser elevada en pacientes con retraso en el diagnóstico, antibioterapia inadecuada y presencia de otros padecimientos crónicos (diabetes, obesidad, tabaquismo, etc) (Azad y Beard, 1998; Faccini-Martínez et al. 2014).

En nuestro país se ha reportado la presencia únicamente de seis especies de rickettsias (Labruna et al. 2011): *Rickettsia amblyommii* en garrapatas *Amblyomma cajennense* sensu lato (*A. cajennense* s.l.) de Veracruz (Medina-Sánchez, 2013; Sosa-Gutierrez et al. 2015); *Rickettsia akari*, *Rickettsia felis* y *Rickettsia typhi* en humanos y

cánidos en Yucatán; *Rickettsia prowazekii* causante de casos de tifo epidémico el estado de Chiapas y en garrapatas *A. cajennense* s.l. y *A. imitator* en Nuevo León (Medina-Sánchez et al., 2005); *Rickettsia rickettsii* que genera la Fiebre Manchada de las Montañas Rocosas en pacientes (en particular en Mexicali, Baja California) (Field-Cortazares et al. 2015) y garrapatas *A. americanum* (Sosa-Gutierrez et al. 2015), *A. imitator* (Oliveira et al. 2010), *A. maculatum* (Sosa-Gutierrez et al. 2015), *A. cajennense* s.l. (Bustamante y Varela, 1946; Sosa-Gutierrez et al. 2015), *A. parvum* (Dzul-Rosado et al. 2013), *Dermacentor nitens* (Sosa-Gutierrez et al. 2015) y *Rhipicephalus sanguineus* (Bustamante y Varela, 1944; Hoffmann, 1962; Eremeeva et al. 2011; Peniche-Lara et al. 2015; Sosa-Gutierrez et al. 2015) en la Comarca Lagunera y el Norte del país (Álvarez Hernández, 2010; Eremeeva et al. 2011; Dzul-Rosado et al. 2013).

Pese a la existencia de estos reportes de casos, se desconoce la epidemiología de las Rickettsiosis a nivel nacional, así como su distribución geográfica en el país. Por lo tanto el objetivo del presente estudio fue identificar el panorama epidemiológico de las Rickettsiosis y elaborar un mapa de distribución de los casos humanos.

Material y Métodos

Se analizaron los datos de morbilidad de las Rickettsiosis en México durante el periodo comprendido entre 2000-2013. Para ello se analizaron los casos confirmados reportados por la Secretaria de Salud provenientes del Sistema de Información en Salud (SINAIS) (CENAPRECE, 2016). Las bases no incluyen los nombres o datos de identificación personales de cada paciente.

Se realizó un análisis descriptivo de los datos, de acuerdo con el grupo etario, el sexo y las entidades administrativas (estado) que notifican el caso. Para ello se realizó un análisis de frecuencias simples. Se obtuvieron los datos geográficos de las localidades donde los casos han sido reportados y se georreferenciación con base en los datos del Catálogo Único de Claves de Áreas Geoestadísticas Estatales, Municipales y Localidades del Instituto Nacional de Estadística y Geografía (INEGI, 2016). Con base en estos datos se generó un mapa de distribución mediante el uso del programa DIVA GIS ver 5.0 (Hijmans et al. 2004).

Este estudio fue aprobado por el Comité de Ética e Investigación de la Facultad de Medicina de la UNAM (Universidad Nacional Autónoma de México) (Fmed/CI/JMO/ 004/2012).

Resultados

Durante el periodo comprendido entre 2000-2013 se identificó la presencia de 1,297 casos de Rickettsiosis, registrados en 31 entidades federativas, concentrándose la mayoría en los estados de Sonora 316 (24.4%), Sinaloa 265 (20.4%) y Guerrero 155 (12%), careciendo de registros únicamente el estado de Morelos (Fig. 1).

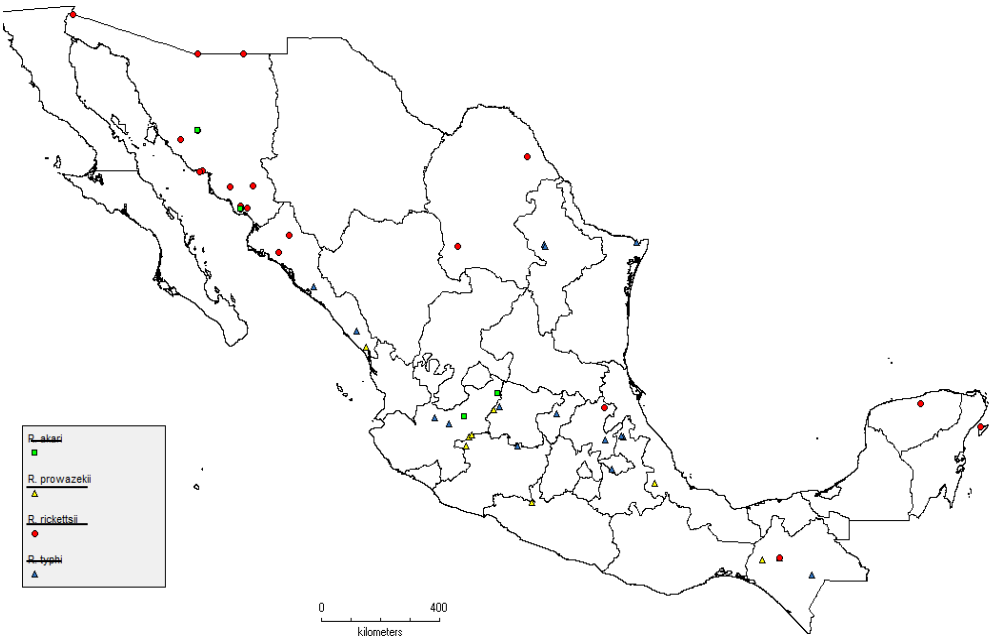

Fig. 1 Distribución de los casos de Rickettsiosis en México durante el periodo 2000-2013.

La notificación de los casos se ha incrementado de manera constante a lo largo del periodo, concentrándose en 2013 con 196 casos (15.1%), seguido por 2012 con 136 casos (10.5%). En contraste en el 2000 únicamente se registraron 27 (2.1%) (Fig. 2). La edad de los pacientes osciló en un rango <1-97 años, con un promedio de 24 años (Fig. 3). Por otro lado 741 (57%) de los casos se presentaron en mujeres y 556 (43%) en hombres en una proporción de 1:1 (Fig. 4).

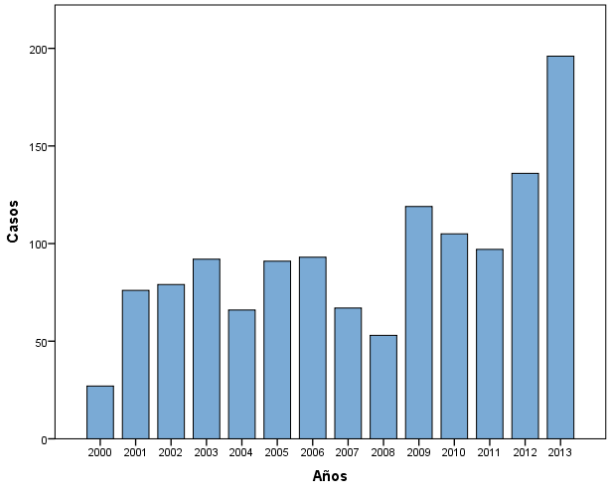

Fig. 2 Casos de Rickettsiosis en México registrados durante el periodo 2000-2013.

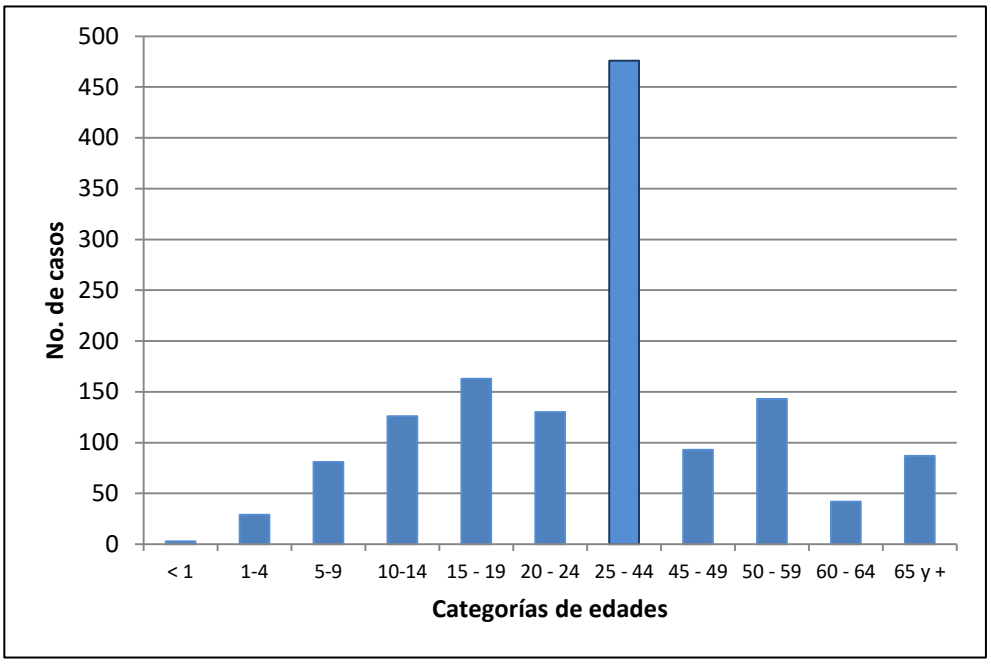

Fig. 3 Distribución de casos de Rickettsiosis por categorías de edades en México durante el periodo 2000-2013.

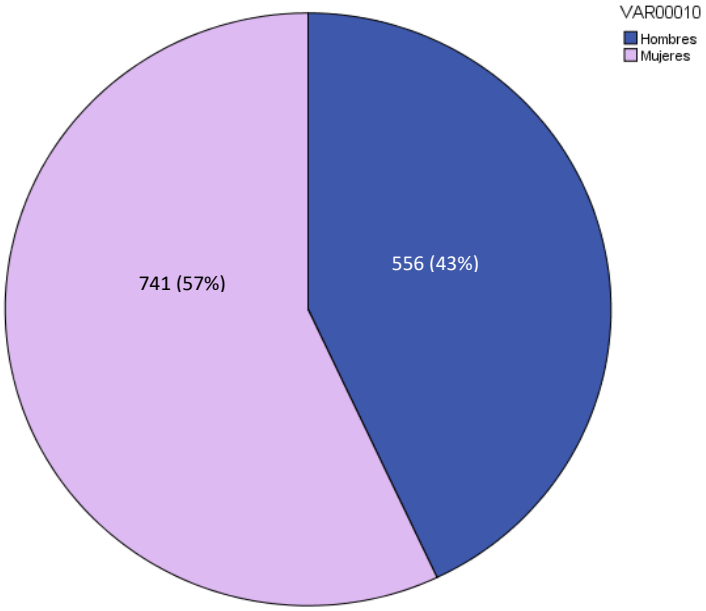

Fig. 4 Distribución de los casos de Rickettsiosis por sexo.

Con relación a la estancia intrahospitalaria 11 (0.8%) de los casos únicamente asistió a consulta externa y no permaneció en la institución, en contraste con 920 (70.9%) los cuales permanecieron entre 1-5 días internados. Sin embargo, también se cuenta con 14 registros que permanecieron entre 27 y 95 días de internalización.

En cuanto a la clasificación de los casos, en 1,186 (91%) de los mismos no se identificó al agente etiológico, registrándose como Tifo no especificados (A75.9) u otras Rickettsiosis (A79). Únicamente en 110 de ellos (9%) pudo identificarse al agente etiológico (5 *R. akari* (A79.1), 8 *R. prowazekii* (A75.0), 80 *R. rickettsii* (A77.0) y 17 *R. typhi* (A75.2) (Fig. 5).

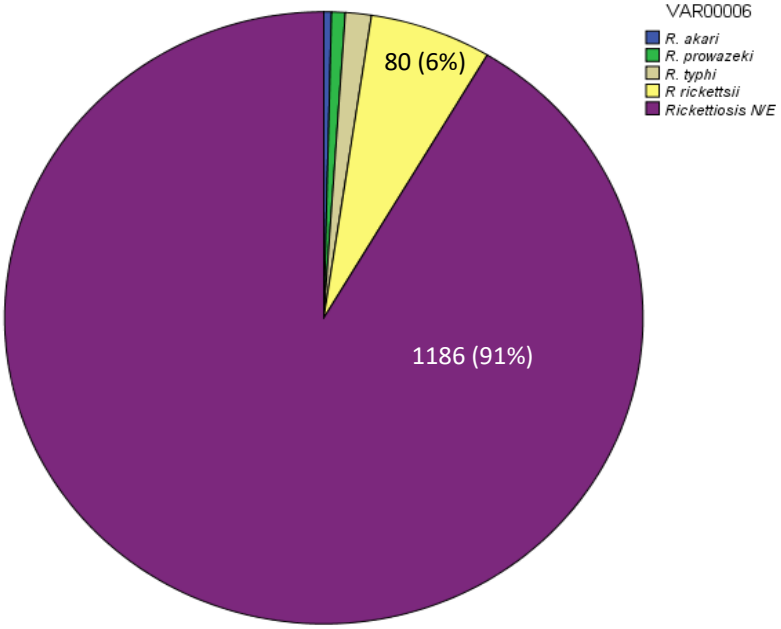

Fig. 5 Distribución de casos de Rickettsiosis por agente etiológico.

Discusión y Conclusiones

En este trabajo mostramos la primera compilación de casos de Rickettsiosis en México, obtenidos a partir de una fuente oficial. Pese a que las Rickettsiosis son enfermedades de notificación obligatoria, la confirmación de un caso resulta compleja, ya que se necesita de muestras pareadas con diferencia de 15 días (InDRE, 2015), y muchos de los casos son fulminantes o el paciente no regresa a la institución hospitalaria, aunado al hecho de que la IFI tiene reacción cruzada entre las especies de *Rickettsia*, por lo cual el número real de pacientes así como las especies causantes siguen siendo aún desconocidos.

Los trabajos previos hacen énfasis en las Rickettsiosis como enfermedades endémicas de zonas como la Comarca Lagunera y la Península de Yucatán (Bustamante y Varela, 1944; Hoffmann, 1962; Eremeeva et al. 2011; Dzul-Rosado et al. 2013; Peniche-Lara et al. 2015), sin embargo, con los datos analizados fue posible identificar que son enfermedades ampliamente distribuidas en el país y que deben de contar con vigilancia activa en cada uno de los estados, principalmente hacia el Centro del país y el Bajío.

Nuestros datos muestran, que la morbilidad de estas enfermedades se ha incrementado durante los últimos años. Esto es posiblemente debido a dos razones: 1) un brote de Fiebre Manchada causada por *R. rickettsii* en Mexicali, Baja California detectado desde 2008 y monitoreado por las autoridades locales (Field-Cortazares et al. 2015) y 2) el establecimiento de 10 laboratorios estatales (certificados para el diagnóstico de las Rickettsiosis) integrados a la Red Nacional de Laboratorios de Salud Pública lo que aumentó el número de muestras analizadas (InDRE, 2015).

Por otro lado, se considera que las Rickettsiosis son enfermedades profesionales de los varones (Faccini-Martinez et al. 2014). Sin embargo, en México no se observa esta tendencia. En cuanto a la edad, el grupo más afectado es de adultos jóvenes de 25-44 años, lo cual supone población económicamente activa, que en conjunto con la hospitalización que es recurrente en más del 70% de los casos, hace pensar que estas enfermedades tienen un elevado costo tanto para el paciente y la familia como para el estado que provee de los servicios de atención.

Por tal motivo resulta de vital importancia la vigilancia epidemiológica activa de las Rickettsiosis en México, ya que constituyen un problema de salud pública que de no atenderse a la brevedad verá incrementado el número de casos por la dispersión de los

vectores por la acción del cambio climático y las condiciones de crecimiento poblacional desmedido que ponen en contacto a un mayor número de personas con reservorios y vectores del medio silvestre.

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Capítulo 3 Diversity of flea-borne and louse-borne pathogens associated with small mammals in murine typhus foci of the centre of Mexico

Diversity of flea-borne and louse-borne pathogens associated with small mammals in murine typhus foci of the centre of Mexico

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Abstract

Fleas and sucking lice are important vectors of multiple pathogens causing major epidemics worldwide, such as plague (*Yersinia pestis*) and epidemic typhus (*Rickettsia prowazekii*). Despite the historical importance of both diseases, this group of ectoparasites has been little studied with respect to other vectors such as mosquitoes or ticks. However these insects have been incriminated as vectors of a wide range of largely understudied and unattended pathogens, especially several species of bacteria's of the genera *Bartonella* and *Rickettsia*. For this reason the aim of the present work was to identify the presence and diversity of *Bartonella* and *Rickettsia* species in endemic murine typhus foci in Hidalgo, Mexico. A

cross-sectional study was carried out to collect hosts and their associated ectoparasites during October, 2014. A total of 47 fleas and 172 sucking lice, belonging to five families (Ceratophyllidae, Leptopsyllidae, Ctenophtalmidae, Hoplopleuridae, Polyplacidae) and related to six species were collected from 42 rodents of four species (*Mus musculus*, *Peromyscus beatae*, *Rattus norvergicus*, *Reithrodontomys sumichrasti*) and one shrew (*Sorex orizabae*). Samples of liver and ear of hosts and entire ectoparasites were fixed in absolute ethanol and examined to identify the presence of *Bartonella* and *Rickettsia* DNA by the amplification of specific fragments of the *gltA* and *ompB* genes. Only four hosts were positive to *Bartonella elizabethae*, *Bartonella vinsonii* and *Rickettsia typhi*. In the case of ectoparasites, 23 specimens of two flea species (*Peromyscopsylla hesperomys* and *Plusaetis mathesoni*) tested positive for *B. vinsonii*. No evidence of *Bartonella* or *Rickettsia* was detected in any lice. Our findings represent the first record of *Bartonella elizabethae,* a confirmed zoonotic pathogen causing endocarditis, in Mexico and several new associations of *Bartonella* with Mexican flea species.

Keywords: *Bartonella elizabethae*, *Rickettsia typhi*, Vectors, Rodents, Emerging diseases

Introduction

Fleas and sucking lice are important vectors of multiple pathogens causing major epidemics worldwide, such as plague (*Yersinia pestis*) and epidemic typhus (*Rickettsia prowazekii*). Despite the historical importance of both diseases, this group of ectoparasites has been little studied with respect to other vectors such as mosquitoes or ticks (Gillespie et al. 2009; Bitam et al. 2010; Eisen & Gage, 2012). However, these groups of insects are hosts for a wide range of largely understudied pathogens, especially several species of bacteria of the genera *Bartonella* and *Rickettsia* (Bitam et al. 2010). The genus *Bartonella* includes at least 33 species of Gram-negative, intracellular and slow-growing coccobacilli with complex life cycles including multiple vertebrate hosts and vectors, such as *Bartonella elizabethae* and *Bartonella vinsonii arupensis,* declared pathogens which cause endocarditis in humans and dogs (Breitschwerdt & Kordick, 2000; Tsai et al. 2011; Kosoy et al. 2012; Regier et al. 2016). On the other hand, *Rickettsia* encompasses 26 species of obligate intracellular bacteria, which are transmitted by different groups of hematophagous arthropods such as

ticks, lice and fleas (Fournier & Raoult, 2009; Merhej et al. 2014). Rickettsia species are classified into four groups, two of which are pathogens for man: members of the Spotted Fever group [SFG] (*R. conorii*, *R. massiliae*, *R. rickettsii*, and *R. parkeri*) and Typhus group [TG] (*R. prowazekii* and *R. typhi*), this latter group transmitted exclusively by lice and fleas, which cause epidemic and murine typhus (Fournier et al. 2003; Fournier & Raoult, 2009).

In recent decades with the advent of molecular biology techniques, the number of species or strains of both bacteria genera has increased exponentially. Particularly, fleas and sucking lice associated with rodents are the groups in which more studies have focused for the detection of pathogens, with the identification of 17 validated species of *Bartonella*, seven of *Rickettsia* and more than 15 new linages for both genera, associated with 49 flea species and seven sucking lice in 25 countries around the world (Table 1).

In Mexico, seven species of fleas (*Ctenocephalides felis*, *Meringis parkeri*, *Orchopeas leucopus*, *Orchopeas sexdentatus*, *Pleochaetis exilis*, *Pulex* sp., and *Polygenis odiosus*) and two of sucking lice (*Hoplopleura hirsuta* and *Polyplax spinulosa*) tested positive for at least one of four validated species of *Bartonella* (*B. vinsonii* and *B. washoensis*) and *Rickettsia* (*R. felis* and *R. prowazekii*). Additionally new lineages of *Bartonella* have been registered in six more flea species (*Echinophaga gallinacea*, *Meringis altipecten*, *Meringis arachis*, *Meringis parkeri*, *Pleochaetis exilis*, *Thrassis aridis*) (Table 1). These records came from isolated studies carried out in wildlife from the southeast and north of the country. Data regarding the center of the country, where there is a report of human cases of murine typhus, are lacking (CENAPRECE, 2016). Additionally in our country there is a record of 172 species of fleas and 44 species of sucking lice, for which reason the inventory of species of both bacteria genera is still far from complete (Acosta-Gutiérrez, 2014; Sánchez Montes et al. 2013).

Due to the great diversity of lice and fleas registered in the country, the hematophagous habits of these ectoparasites, the fact that multiple species of both bacterial genera are transmitted by the same vector species and can co-infect the same groups of hosts, the possibility of hosts to establish near human settlements, and the historical presence of human cases of murine typhus in the centre of the country, this study was carried out to

identify the presence and diversity of *Bartonella* and *Rickettsia* species in a foci of murine typhus in Hidalgo, Mexico.

Material and methods

During August to September 2014 we performed a field trip in two private ranches from Mineral del Monte and Tulancingo de Bravo, of the state of Hidalgo, Mexico, close to sites where human murine typhus cases have been reported (CENAPRECE, 2016). This study was approved by the Ethics and Research Committee of the Medical Faculty of the UNAM [FMED/CI/JMO/004/2012] (Universidad Nacional Autónoma de México).

In order to identify the presence of several flea-borne and louse-borne pathogens (*Rickettsia* and *Bartonella*) in small mammals and their associated ectoparasites, we trapped small mammals using Sherman traps with methods previously referred (Romero-Almaraz et al. 2007), under permission FAUT-0170 from the Secretaría del Medio Ambiente y Recursos Naturales. All mammals were sacrificed in accordance with the Guidelines of the American Society of Mammalogists for the Use of Wild Mammals in Research (Gannon & Sikes, 2007). We performed the necropsy of each animal, extracting a portion of liver and ear, which were fixed in 96% ethanol until processing in the laboratory. Additionally, fleas and lice were recovered from host's bodies by manual inspection and fixed in absolute ethanol. Hosts were identified and deposited at Museo de Zoología "Alfonso L. Herrera" Facultad de Ciencias (MZFC) and Colección del Centro de Medicina Tropical, Facultad de Medicina (CMTFM), both belonging to Universidad Nacional Autónoma de México.

For morphological determination, fleas and lice were mounted on slides using the modified techniques of Kim et al. (1986) and Wirth and Marston (1968). Species were identified using specialized keys such as of Kim et al. (1986) for lice and Acosta and Morrone (2003), Hastriter (2004), Hopkins and Rothschild (1971), Morrone et al. (2000), and Traub (1950) for fleas.

From collected ectoparasites and hosts tissues, we extracted DNA with the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). As internal control of the extraction and for molecular identification of the ectoparasites, we amplified a fragment of 400 bp of Cytochrome Oxidase Subunit I (COI) gene. For pathogens detection, we amplified several fragments of *gltA* and *ompB* genes specific for each group (Table 2).

The reaction mixture consisted of 12.5 µL of GoTaq® Green Master Mix, 2X of Promega Corporation (Madison, WI, USA), the pair of primers (100 ng each), 6.5 µL nuclease-free water and 30 ng DNA in a final volume of 25 µL (Sánchez-Montes et al. 2016 a,b). PCR products were resolved in 2% agarose gels using TAE buffer at 85V during 45 minutes and visualized using an ODYSSEY CLx Imaging System (LICOR Biosciences). Purified amplification products were submitted for sequencing at Macrogen Inc., Korea. Sequences were analysed and edited using Bioedit and deposited in GenBank under accession numbers (Submitted). In order to identify the species of *Bartonella* and *Rickettsia*, we used the similarity criteria of the *gltA* and *ompB* genes proposed by La Scola (2003), Fournier & Roult (2009) and Fournier et al. (2003). Global alignments were done using Clustal W and the best substitution model was selected based on the lowest BIC (Bayesian Information Criterion) score for each gene using MEGA 6.0 (Sánchez-Montes et al. 2016c). Additionally phylogenetic reconstruction was done using Maximum Likelihood and the support of the branches was evaluated over 10,000 bootstrap replications.

Results

We collected 42 rodents from four species (*Mus musculus*, *Peromyscus beatae*, *Rattus norvergicus*, and *Reithrodontomys sumichrasti*), and one shrew (*Sorex orizabae*). We detected the presence of *Bartonella* DNA in four samples of liver of two *Peromyscus beatae* (2/28=7%) and two *Rattus norvergicus* (2/4=50%). Sequences recovered from *P. beatae* exhibited a homology of 98% with *Bartonella vinsonii vinsonii* (a member of the *Bartonella vinsonii* complex) and those from *Rattus norvergicus* showed 100% homology with *Bartonella elizabethae* (Fig. 1A). In the case of *Rickettsia* detection, a single specimen of *Rattus norvergicus* (1/4=25%) tested positive in samples from liver and ear. Sequences of *gltA* and *ompB* genes exhibited a similarity of 99% and 100% with *Rickettsia typhi* (Accesion number AE017197) deposited in Genbank (Fig. 1B). A single *Rattus norvergicus* specimen had co-infections of *Bartonella elizabethae* and *Rickettsia typhi*. The hosts were infested by 47 fleas (15♀, 24♂, 8 undetermined) and 172 sucking lice (60♀, 39♂, 73 nymphs), which were distributed in six species belonging to five families and six genera (Table 3). No fleas or lice were recovered from *Mus musculus* and *Sorex orizabae*. After morphological identification was done, we amplified a fragment of 400 bp

of Cytochrome oxidase subunit I (COI) in all the recovered ectoparasites in order to corroborate the identification of all samples, especially of damaged specimens and nymphal stages. COI sequences of five of the six species analysed, were deposited in Genbank with the following accession numbers: *C. tecpin* (SUBMITTED), *P. hesperomys adelpha* (SUBMITTED), *P. mathesoni* (SUBMITTED), *H. reithrodontomydis* (SUBMITTED) and *P. spinulosa* (SUBMITTED). No complete sequences were obtained for *J. breviloba breviloba.* We detected the presence of the same *Bartonella* lineage previously refereed in *P. beatae*, in two flea species (6 *P. hesperomys adelpha* and 17 *P. mathesoni*) recovered from the two hosts, which tested positive and from three others that were negative (Table 3). Sequences from fleas and hosts shape a single cluster within our phylogenetic analysis (Fig. 1A). None of the flea or sucking lice species analysed were positive for *Rickettsia* DNA.

Discussion

We report for the first time the presence of two species of *Bartonella* and one *Rickettsia* in the state of Hidalgo, Mexico. The first *Bartonella* species is a member of the *Bartonella vinsonii* complex, closely related to previous sequences detected in Cricetid rodents and fleas of the North of the country (Rubio et al. 2014; Fernández-González et al. 2016). Also, this is the first study to report the presence of a *Bartonella* in the fleas *Peromyscopsylla hesperomys adelpha* and *Plusaetis mathesoni* and the host *Peromyscus beatae.* Our phylogenetic analysis grouped sequences of *B. vinsonii* from *P. hesperomys adelpha*, *P. mathesoni*, and *P. beatae* in a single cluster, which is the reason for considering that both species of fleas could be their potential vectors. Additionally positive *P. hesperomys adelpha* were recovered from negative hosts, suggesting that these fleas may disseminate the pathogen in non-infected individuals among the rodent population bacteria (Kosoy et al. 1997; Morick et al. 2010). However, it is necessary to carry out tests to verify their vectorial capacity. Both species of fleas have a restricted distribution in Mexico, which extend to northeast and center of the country, parasitizing several cricetid species such as *Peromyscus levipes, Peromyscus maniculatus, Reithrodontomys megalotis* (*P. mathesoni*) and *Peromyscus difficilis* (*P. hesperomys adelpha*), so it is not unexpected that this strain of

bacteria is widely distributed in the country (Ponce-Ulloa & Llorente-Bousquets, 1993; Hoffman et al. 1989; Whitaker & Morales-Malacara, 2005; Acosta & Fernández, 2015). We also report for the first time the presence of *B. elizabethae* in Mexico, a zoonotic bacterial, which causes endocarditis and neuroretinitis in humans. This agent was reported for the first time in the 1990s in the USA, and become an emerging problem in several countries of Southeast Asia, Portugal and France (Regier et al. 2016; Tay et al. 2016). *B. elizabethae* is mainly transmitted by rat fleas *Xenosylla cheopis*. However, in our study we did not recover any fleas from the four *R. norvergicus* analysed. The higher prevalence of *B. elizabethae* in collected murid rodents suggests the presence of this flea or other competent vector in the zone (Bitam et al. 2012). Additionally, we demonstrated for the first time the presence of *Rickettsia typhi* in rodents of the state of Hidalgo. This *Rickettsia* produces febrile illness with a wide range of severity, in particular in infants that could lead to systemic failure (Zavala-Castro et al. 2009). In the state of Hidalgo, three cases of murine typhus had been reported between 2005-2010, yet an outbreak with 12 cases was registered in 2015 (CENAPRECE, 2016). The detection of positive rodents in addition to the presence of new human cases suggests that conditions for the complete life cycle of this pathogen possibly exist in this area.

Only one rat reported coinfection by *B. elizabethae* and *R. typhi*, a phenomenon that has been previously reported, probably because both pathogens are transmitted by the same flea species, that reinforces the theory of the presence of this vector in the study area (Marie et al. 2006; Bitam et al. 2012; Frye et al. 2015). The presence of positive Norway rats for these two zoonotic pathogens poses a risk to human health, because this rodent species invades suburban and urban areas, settling into human settlements. Furthermore, these rats could be carrying fleas that can feed on humans leading to urban outbreaks.

Taken together, we here show the first records of several confirmed zoonotic pathogens that can cause murine typhus and endocarditis in Mexico, which highlight the importance of the establishing active entomological surveillances in wildlife.

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FIGURE LEGENDS

Figure 1a. Maximum likelihood (ML) phylogenetic tree generated with *gltA* gene from several members of the genus *Bartonella*. The nucleotide substitution model was the Tamura three parameter model (T92) with discrete Gamma distribution (+G). Bootstrap values higher than 50 are indicated at the nodes. Sequences recovered in the study are marked with solid rhombuses and triangles.

Figure 1b. Maximum likelihood (ML) phylogenetic tree generated with *gltA* and *ompB* genes concatenated from several members of the genus *Rickettsia*. The nucleotide

substitution model was the Tamura three parameter model (T92) with discrete Gamma distribution (+G). Bootstrap values higher than 50 are indicated at the nodes. Sequences recovered in the study are marked with solid triangles.

Table 1. *Bartonella* and *Rickettsia* species detected in fleas and sucking lice associated with rodents worldwide.

Bacteria species	Flea	Host	Country	References
B. birtlesii	*Ctenophthalmus andorrensis catalanensis*	*Apodemus sylvaticus*	Spain	Cevidanes et al. 2017
	Leptopsylla taschenbergi amitina	*Apodemus sylvaticus*	Spain	Cevidanes et al. 2017
B. coopersplainsensis	*Stephanocircus pectinipes*	*Rattus fuscipes*	Australia	Kaewmongkol et al. 2011
B. doshiae	*Xenopsylla cheopis*	*Rattus* sp.	Afghanistan	Marie et al. 2006
B. elizabethae	*Leptopsylla segnis*	*Mus spretus*	Algeria	Bitam et al. 2012
	Synosternus cleopatrae	*Gerbillus pyramidum*	Israel	Morick et al. 2010
	Synopsyllus fonquerniei	*Rattus rattus*	Madagascar	Brook et al. 2017
	Xenopsylla cheopis	*Rattus norvergicus*	Algeria, USA	Bitam et al. 2012; Frye et al. 2015
		Rattus rattus	Algeria	Bitam et al. 2012
		Rattus tanezumi	Indonesia	Winoto et al. 2005
		Rattus sp.	Afghanistan	Marie et al. 2006
			Nigeria	Kamani et al. 2013

B. grahamii	*Ctenophthalmus agyrtes*	ND	Lithuania	Lipatova et al. 2015
	Ctenophthalmus andorrensis catalanensis	*Apodemus sylvaticus*	Spain	Cevidanes et al. 2017
		Crocidura russula		
	Ctenophthalmus nobilis	*Clethrionomys glareolus*	England	Bown et al. 2004
	Megabothris turbidus	ND	Lithuania	Lipatova et al. 2015
	Megabothris walker	ND	Lithuania	Lipatova et al. 2015
	Synosternus cleopatrae	ND	Israel	Rzotkiewicz et al. 2015
	Xenopsylla ramesis	ND	Israel	Rzotkiewicz et al. 2015
B. henselae	*Xenopsylla ramesis*	ND	Israel	Rzotkiewicz et al. 2015
		Meriones tristrami	Israel	Morick et al. 2010
B. koehlerae	*Xenopsylla gerbilli*	*Meriones lybicus*	Afghanistan	Marie et al. 2006
B. phoceensis	*Xenopsylla cheopis*	*Rattus tanezumi*	Indonesia	Winoto et al. 2005
B. queenslandensis	*Xenopsylla cheopis*	*Rattus* sp.	*Thailand*	Klangthong et al. 2015
B. quintana	*Xenopsylla gerbilli*	*Meriones lybicus*	Afghanistan	Marie et al. 2006

B. rattaustraliani	*Stephanocircus dasyure*	*Rattus fuscipes*	Australia	Kaewmongkol et al. 2011
B. rattimassiliensis	*Xenopsylla cheopis*	*Rattus tanezumi*	Indonesia	Winoto et al. 2005
B. rochalimae	*Xenopsylla cheopis*	*Rattus norvergicus*	USA	Frye et al. 2015
B. taylorii	*Ctenophthalmus agyrtes*	ND	Lithuania	Lipatova et al. 2015
	Ctenophthalmus andorrensis catalanensis	*Apodemus sylvaticus, Crocidura russula, Mus spretus*	Spain	Cevidanes et al. 2017
	Ctenophthalmus nobilis	*Clethrionomys glareolus*	England	Bown et al. 2004
	Ctenophthalmus uncinatus	ND	Lithuania	Lipatova et al. 2015
	Hystrichopsylla talpae	ND	Lithuania	Lipatova et al. 2015
	Leptopsylla taschenbergi amitina	*Apodemus sylvaticus*	Spain	Cevidanes et al. 2017
	Megabothris turbidus	ND	Lithuania	Lipatova et al. 2015
	Megabothris walkeri	ND	Lithuania	Lipatova et al. 2015

	Xenopsylla gerbilli	*Meriones lybicus*	Afghanistan	Marie et al. 2006
B. tribocorum	*Ctenophthalmus* sp.	*ND*	Nigeria	Kamani et al. 2013
	Xenopsylla cheopis	*Rattus norvergicus*	USA	Reeves et al. 2007a; Frye et al. 2015
		Rattus rattus	Algeria	Bitam et al. 2012
		Rattus tanezumi flavipectus	China	Li et al. 2007
		Rattus sp.	Thailand	Klangthong et al. 2015
B. vinsonii	*Polygenis gwyni*	*Sigmodon hispidus*	USA	Abbot et al. 2007
B. vinsonii arupensis	*Orchopeas leucopus*	*Peromyscus leucopus, Peromyscus maniculatus*	Mexico	Fernández-González et al. 2016
B. vinsonii vinsonii	*Ctenophthalmus pseudagyrtes*	*Microtus* sp.	USA	Reeves et al. 2007[a]
	Meringis parkeri	*Onychomys arenicola, Onychomys leucogaster*	Mexico	Fernández-González et al. 2016
	Orchopeas sexdentatus	*Neotoma albigula*	Mexico	Fernández-González et al. 2016
	Pleochaetis exilis	*Neotoma albigula,*	Mexico	Fernández-González et al.

		Onychomys arenicola, *Onychomys leucogaster*, *Peromyscus maniculatus*		2016
B. washoensis	*Orchopeas hirsuta*	*Cynomys* sp.	USA	Stevenson et al. 2003; Reeves et al. 2007b
	Orchopeas howardi	*Sciurus carolinensis*	USA	Durden et al. 2004
	Pulex sp.	*Cynomys ludovicianus*	Mexico	Fernández-González et al. 2016
	Thrassis fotus	*Cynomys* sp.	USA	Reeves et al. 2007b
Bartonella near *birtlesii*	*Orchopeas howardi*	*Sciurus carolinensis*	USA	Reeves et al. 2006b
Bartonella near *clarridgeiae*	*Ctenophthalmus lushuiensis*	*Eothenomys spp.*	China	Li et al. 2007
	Leptopsylla segnis	*Rattus rattus*	Egypt	Loftis et al. 2006
	Polygenis gwyni	*Sigmodon hispidus*	USA	Abbot et al. 2007
Bartonella near *doshiae*	*Ctenophthalmus andorrensis catalanensis*	*Apodemus sylvaticus*	Spain	Cevidanes et al. 2017
	Leptopsylla	*Apodemus*	Spain	Cevidanes et al.

	taschenbergi amitina	*sylvaticus*		2017
Bartonella near *elizabethae*	*Ctenophthalmus andorrensis catalanensis*	*Apodemus sylvaticus*	Spain	Cevidanes et al. 2017
	Leptopsylla algira	ND	Israel	Rzotkiewicz et al. 2015
		Mus musculus	Israel	Morick et al. 2010
	Leptopsylla taschenbergi amitina	*Apodemus sylvaticus*	Spain	Cevidanes et al. 2017
	Ornithophaga sp.	*Mus spretus*	Portugal	De Sousa et al. 2006
	Stenoponia tripectinata	*Mus spretus*	Portugal	De Sousa et al. 2006
		Rattus rattus	Portugal	De Sousa et al. 2006
	Synosternus cleopatrae	ND	Israel	Rzotkiewicz et al. 2015
		Gerbillus pyramidum	Israel	Morick et al. 2010
	Xenopsylla cheopis	*Rattus* sp.	Thailand	Klangthong et al. 2015
	Xenopsylla ramesis	ND	Israel	Rzotkiewicz et al. 2015

Bartonella near *grahamii*	*Meringis altipecten*	*Onychomys arenicola, Onychomys leucogaster, Dipodomys merriami*	Mexico	Fernández-González et al. 2016
	Meringis arachis	*Onychomys arenicola, Onychomys leucogaster, Dipodomys merriami*	Mexico	Fernández-González et al. 2016
	Meringis parkeri	*Onychomys arenicola, Onychomys leucogaster, Dipodomys merriami*	Mexico	Fernández-González et al. 2016
	Pleochaetis exilis	*Onychomys arenicola, Onychomys leucogaster*	Mexico	Fernández-González et al. 2016
	Synosternus cleopatrae	*Meriones sacramenti*	Israel	Morick et al. 2010
	Xenopsylla ramesis	ND	Israel	Rzotkiewicz et al. 2015
Bartonella near *henselae*	*Orchopeas howardi*	*Glaucomys volans*	USA	Reeves et al. 2007a
	Synosternus	*Gerbillus andersoni*	Israel	Morick et al.

	cleopatrae	*allenbyi*		2010
Bartonella near *phoceensis*	*Xenopsylla cheopis*	*Rattus norvergicus, Rattus rattus*	Egypt	Loftis et al. 2006
Bartonella sp. near quintana	*Orchopeas howardi*	*Sciurus carolinensis*	USA	Durden et al. 2004
Bartonella near rochalimae	*Leptopsylla taschenbergi amitina*	*Apodemus sylvaticus*	Spain	Cevidanes et al. 2017
	Xenopsylla cheopis	*Rattus norvegicus*	Algeria	Bitam et al. 2012
	Xenopsylla ramesis	ND	Israel	Rzotkiewicz et al. 2015
Bartonella near *taylorii*	*Ctenophthalmus lushuiensis*	*Eothenomys spp.*	China	Li et al. 2007
Bartonella near *tribocorum*	*Xenopsylla cheopis*	*Rattus rattus*	Benin	Leulmi et al. 2014
Bartonella near *vinsonii arupensis*	*Synosternus cleopatrae*	ND	Israel	Rzotkiewicz et al. 2015
Bartonella sp.	*Echinophaga gallinacea*	*Dipodomys spectabilis*	Mexico	Fernández-González et al. 2016
	Ctenophthalmus andorrensis catalanensis	*Crocidura russula*	Spain	Cevidanes et al. 2017
	Meringis arachis	*Dipodomys spectabilis*	Mexico	Fernández-González et al.

				2016
	Meringis altecpin	*Dipodomys spectabilis, Onychomys arenicola*	Mexico	Fernández-González et al. 2016
	Orchopeas hirsuta	*Cynomys* sp.	USA	Reeves et al. 2007b
	Synosternus cleopatrae	ND	Israel	Rzotkiewicz et al. 2015
	Thrassis aridis	*Dipodomys spectabilis*	Mexico	Fernández-González et al. 2016
	Xenopsylla cheopis	*Rattus norvegicus*	Algeria	Bitam et al. 2012
		Rattus rattus	Algeria, Israel	Morick et al. 2010; Bitam et al. 2012
R. conorii	*Stivalius aporus*	*Mus caroli*	Taiwan	Kuo et al. 2016
R. felis	*Acropsylla episema*	*Apodemus agrarius*	Taiwan	Kuo et al. 2016
	Anomiopsyllus nudata	*Neotoma albigula*	USA	Stevenson et al. 2003
	Ctenocephalides felis	*Peromyscus yucatanicus*	Mexico	Peniche Lara et al. 2015
		Rattus norvegicus	Cyprus	Psaroulaki et al. 2006
		Rattus rattus	Cyprus	Psaroulaki et al.

				2006
	Ctenophthalmus calceatus calceatus	*Lophuromys aquilus*	Tanzania	Leulmi et al. 2014
	Ctenophthalmus sp.	*Rattus norvegicus*	Portugal	De Sousa et al. 2006
	Leptopsylla segnis	*Mus* sp.	Algeria.	Bitam et al. 2009
	Polygenis odiosus	*Ototylomys phyllotis*	Mexico	Peniche Lara et al. 2015
	Stivalius aporus	*Mus caroli*	Taiwan	Kuo et al. 2016
	Xenopsylla cheopis	*Rattus norvegicus*	Cyprus	Christou et al. 2010
		Rattus rattus	Cyprus, Madagascar	Christou et al. 2010; Rakotonanahary et al. 2017
		Rattus sp.	Afghanistan, Algeria.	Marie et al. 2006; Bitam et al. 2009
R. japonica	*Stivalius aporus*	*Mus caroli*	Taiwan	Kuo et al. 2016
R. raoultii	ND	*Apodemus flavicollis, Myodes glareolus*	Germany	Obiegala et al. 2016
R. typhi	*Ctenophthalmus congeneroides*	*Apodemus agrarius*	South Korea	Kim et al.2010

Leptopsylla segnis	*Rattus norvegicus*	Cyprus	Christou et al. 2010
	Rattus rattus	Cyprus, Egypt, Portugal	De Sousa et al. 2006, Loftis et al. 2006; Christou et al. 2010
Rhadinopsylla insolita	*Apodemus agrarius*	South Korea	Kim et al. 2010
Xenopsylla brasiliensis	*Mastomys natalensis*	Tanzania	Leulmi et al. 2014
	Rattus rattus	Tanzania	Leulmi et al. 2014
	Rattus sp.	Democratic Republic of the Congo	Leulmi et al. 2014
Xenopsylla cheopis	*Rattus norvegicus*	Cyprus, Egypt	Loftis et al. 2006; Christou et al. 2010
	Rattus rattus	Benin, Cyprus, Egypt, Madagascar	Loftis et al. 2006; Christou et al. 2010; Leulmi et al. 2014, Rakotonanahary et al. 2017
	Rattus sp.	Argelia	Bitam et al. 2009

R. prowazekii	*Orchopeas howardii*	*Glaucomys Volans*	USA	Sonenshine et al. 1978
Candidatus Rickettsia Asemboensis	*Echidnophaga gallinacea*	*Rattus rattus*	Egypt	Loftis et al. 2006
	Synosternus cleopatrae	ND	Israel	Rzotkiewicz et al. 2015
	Xenopsylla ramesis	*Gerbillus dasyurus, Meriones tristrami, Mus musculus*	Israel	Rzotkiewicz et al. 2015
Rickettsia felis-like	*Xenopsylla ramesis*	ND	Israel	Rzotkiewicz et al. 2015
Rickettsia near *monacensis*	*Oropsylla hirsuta*	*Cynomys* sp.	USA	Reeves et al. 2007b
Rickettsia sp. Oh16	*Orchopeas howardi*	*Sciurus carolinensis*	USA	Reeves et al. 2006b
Rickettsia sp. TwKM01	*Stivalius aporus*	*Apodemus agrarius*	Taiwan	Kuo et al. 2016
Bacteria species	**Sucking lice**	**Host**	**Country**	**References**
B. henselae	*Neohaematopinus sciuri*	*Sciurus carolinensis*	USA	Durden et al. 2004
B. phoceensis	*Hoplopleura pacifica*	*Rattus norvegicus*	Egypt	Reeves et al. 2006
	Polyplax spinulosa	*Rattus norvegicus*	Taiwan	Tsai et al. 2010

	Polyplax sp.	*Rattus rattus*	Madagascar	Brook et al. 2017
		Rattus sp.	Thailand	Klangthong et al. 2015
B. rattimassiliensis	*Hoplopleura pacifica*	*Rattus norvegicus*	Egypt	Reeves et al. 2006
	Polyplax spinulosa	*Rattus norvegicus*	Egypt, Taiwan	Reeves et al. 2006; Tsai et al. 2010
	Polyplax sp.	*Rattus rattus*	Madagascar	Brook et al. 2017
		Rattus sp.	Thailand	Klangthong et al. 2015
B. tribocorum	*Polyplax spinulosa*	*Rattus norvegicus*	Taiwan	Tsai et al. 2010
B. vinsonii	*Hoplopleura hirsuta*	*Sigmodon hispidus*	Mexico	Sánchez-Montes et al. 2016b
B. washoensis	*Neohaematopinus sciuri*	*Sciurus carolinensis*	USA	Durden et al. 2004
Bartonella near *grahamii*	*Nosopsyllus fasciatus*	*Rattus surifer*	Thai-Myanmar Border	Parola et al. 2003
Bartonella near *tribocorum*	*Polyplax spinulosa*	*Rattus norvegicus*	Egypt	Reeves et al. 2006
Bartonella near *washoensis*	*Hoplopleura sciuricola*	*Sciurus carolinensis*	USA	Durden et al. 2004
Bartonella sp.	*Polyplax* sp.	*Thrichomys apereoides*	Brazil	Fontalvo et al. 2017
R. prowazekii	*Neohaematopinus*	*Glaucomys*	USA	Sonenshine et al.

	sciuropteri	*volans*		1978
	*Polyplax spinulosa**	*Rattus norvegicus*	Mexico	Mooser et al. 1931
R. typhi	*Enderleinellus marmotae*	*Marmota monax*	USA	Reeves et al. 2006b
	Hoplopleura pacifica	*Rattus norvegicus*	Egypt	Reeves et al. 2006a

Table 2. Oligonucleotide primers used in this study.

Target organism	**Gen**	**Primers**	**Sequence (5´-3´)**	**Length (bp)**	**Reference**
Fleas and lice	*COI* (Cytochrome oxidase subunit I)	L6625	CCGGATCCTTYTGRTTYTTYGGNCAYCC	400	Hafner et al. 1994
		H7005	CCGGATCCACNACRTARTANGTRTCRTG		
Rickettsia sp.	*gltA* (Citrate synthase)	RpCS.415	GCTATTATGCTTGCGGCTGT	806	de Souza et al. (2006)
		RpCS.1220	TGCATTTCTTTCCATTGTGC		
	ompB	120-M59	CCGCAGGGTTGGTAACTGC	862	Roux & Raoult, 2000
		120–807	CCTTTTAGATTACCGCCTAA		
Bartonella sp.	*gltA* (Citrate synthase)	BhCS781.p	GGGGACCAGCTCATGGTGG	379	Norman et al. 1995
		BhCS1137.n	AATGCAAAAAGAACAGTAAACA		

Table 3. Diversity and prevalence of *Bartonella* and *Rickettsia* species detected in fleas, sucking lice and small mammals in Hidalgo, Mexico.

Host						**Ectoparasite**					
Family	**Species**	**Nt**	**Ni**	**%**	**BAD**	**Family**	**Species**	**EA**	**EI**	**%**	**BAD**
Cricetidae	*Peromyscus beatae*	28	2	7	*Bartonella vinsonii*	Ceratophyllidae	*Jellisonia breviloba breviloba*	3	0	0	ND
							Plusaetis mathesoni	30	17	57	*Bartonella vinsonii*
						Ctenophtalmidae	*Ctenophthalmus tecpin*	3	0	0	ND
						Leptopsyllidae	*Peromyscopsylla hesperomys adelpha*	7	6	86	*Bartonella vinsonii*
	Reithrodontomys sumichrasti	2	0	0	ND	Hoplopleuridae	*Hoplopleura reithrodontomydis*	4	0	0	ND
Muridae	*Mus musculus*	8	0	0	ND	NR	NR	NR	NR	NR	ND
	Rattus norvergicus	4	2	50	*Bartonella elizabethae*	Polyplacidae	*Polyplax spinulosa*	172	0	0	ND

			1	25	*Rickettsia typhi*							
Soricidae	*Sorex orizabae*	1	0	0	ND	NR	NR	NR	NR	NR	ND	

Nt: Host collected; **Ni**: Number of hosts infected; **BAD**: Bacterial agents detected; **EA**: Ectoparasites abundance; **EI**: Ectoparasites infected; **NR**: Not recovered; **ND**: Not detected; **%**: prevalence

Capítulo 4 First report of "*Candidatus* Rickettsia amblyommii" in west coast of Mexico

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Original article

First report of "*Candidatus* Rickettsia amblyommii" in west coast of Mexico

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ABSTRACT

We report the first case of "*Candidatus* Rickettsia amblyommii" detected in *Amblyomma mixtum* ticks on humans on the west coast of Mexico. This is the most western record of "*Ca.* R. amblyommii" in the Western Hemisphere, representing the first record for the western coast of the Americas. Even if the record is far from the previously known locations for the species it does not represent a new record regarding temperature, precipitation and topographic parameters. Since "*Ca.* R. amblyommii" antibodies have been detected in patients suspected of Rocky Mountain spotted fever, and the tick *A. mixtum* has been associated with humans, it is important to consider "*Ca.* R. amblyommii" as a potential risk for the human population that has not been considered at risk before.

1. Introduction

Ticks are obligate hematophagous ectoparasites that are associated with a wide range of vertebrate hosts. To date, 896 species are recognized worldwide (Guglielmone et al., 2010), and in Mexico only 100 species have been reported (Pérez et al., 2014). Due to their feeding habits and the wide range of hosts that they can parasitize, ticks are important vectors of several pathogens, especially rickettsiae. The genus *Rickettsia* encompasses 26 obligatory intracellular bacteria that infect arthropods and can cause several diseases in vertebrates (Merhej et al., 2014). In Mexico only five species of rickettsiae, that cause diseases in humans, have been reported: a rickettsial pox agent (*Rickettsia akari*), two typhus group agents (*Rickettsia prowazekii*, *Rickettsia typhi*) and two spotted fever group agents (*Rickettsia felis*, *Rickettsia rickettsii*) (Labruna et al., 2011). However, several human cases of spotted fever have been registered without identification of the etiological agent (CENAPRECE, 2015). On the other hand, rickettsial pathogens have been identified in hard ticks including: "*Candidatus* Rickettsia amblyommii" in *Ambyomma cajennense* sensu lato (s. l.) (Medina-Sánchez, 2013; Sosa-Gutierrez et al., 2015); *Rickettsia prowazekii* in *Amblyomma cajennense* s. l. and in *Amblyomma imitator* (Medina-Sánchez et al., 2005); *Rickettsia rickettsii* in *Amblyomma americanum* (Sosa-Gutierrez et al., 2015), *A. imitator* (Oliveira et al., 2010), *Amblyomma maculatum* (Sosa-Gutierrez et al., 2015), *A. cajennense* s. l. (Bustamante and Varela, 1946; Sosa-Gutierrez et al., 2015), *Amblyomma parvum* (Dzul-Rosado et al., 2013), *Dermacentor nitens* (Sosa-Gutierrez et al., 2015) and *Rhipicephalus sanguineus* (Bustamante and Varela, 1944; Eremeeva et al., 2011; Hoffmann, 1962; Peniche-Lara et al., 2015; Sosa-Gutierrez et al., 2015).

Given the increase of recreational activities that are carried out in the wilderness places inhabited by many tick species, it is necessary to identify potential rickettsial agents circulating in wildlife that can infect population groups at risk such as people living

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in those areas, campers, biologists, anthropologists, veterinarians, among others who visit, for example natural parks and natural protected areas. Additionally, the capacity to identify the environmental characteristics associated with the presence of the pathogen and the ticks could be useful for establishing their distribution.

2. Material and methods

During November 2013, we collected ticks attached to people in the hotel SNTE Sección 47 (19.55°N, 105.08°W), located close to Chamela Biological Station (which is part of the Chamela-Cuixmala Biosphere Reserve) in Jalisco, Mexico. Ticks were removed using tweezers and afterwards fixed with 96% ethanol. Twenty-five specimens were collected and morphologically identified using specialized taxonomic keys such as Guzmán-Cornejo et al. (2011) and Nava et al. (2014) for adults and Martins et al. (2010, 2014) for nymphs identification.

After morphological identification, a small incision was made behind the fourth pair of legs of ticks for DNA extraction. We followed the protocol for DNA Tissue extraction of Blood and Tissue Kit (QIAGEN, Hilden, Germany).

In order to identify the presence of rickettsial DNA in the samples analyzed, we amplified a fragment of 800 bp corresponding to citrate synthase gene (*gltA*) according to the specifications of de Sousa et al. (2006). The reaction mixture consisted of 12.5 μl PCR master mix solution (Qiagen), 100 pg of each primer, 6.5 μl nuclease-free water, and 200 ng DNA in a final volume of 25 μl. A negative control (reaction mix without DNA) and a positive (*Rickettsia rickettsii* DNA) were included. Positive samples were characterized by amplification of fragments of 862 bp of outer membrane protein B (*ompB*) and 532 bp of outer membrane protein A (*ompA*) (which recognized spotted fever rickettsia members), using the specific primers and conditions reported by Regnery et al. (1991) and Roux and Raoult (2000). PCR products were resolved in 1.5% agarose gels and visualized in an ODYSSEY CLx Imaging System (LI-COR Biosciences).

Positive PCR products were sequenced upstream at Laboratorio de Biología Molecular y de la Salud, Universidad Nacional Autónoma de México. All sequences were compared with references available in GenBank using Basic Local Alignment Search Tool (BLAST). Additionally, multiple alignments were done using CLUSTAL W and a neighbor-joining phylogenetic tree was generated for each gene with Kimura two-parameter model using Mega 5.01 (Tamura et al., 2011).

We decided to identify the geographic and environmental importance of our record, compiling all the geographic records from published and unpublished literature of the rickettsia species found in the Western Hemisphere (Alves et al., 2014; Barrett et al., 2014; Bermúdez et al., 2009; Budachetri et al., 2014; de Barros-Lopes et al., 2014; Faccini-Martínez et al., 2016; Gaines et al., 2014; Hermance et al., 2014; Hun et al., 2011; Jiang et al., 2010; Killmaster et al., 2014; Labruna et al., 2004a,b; Leydet and Liang, 2013; Lopes et al., 2016; Lugarini et al., 2015; McIntosh et al., 2015; Medeiros et al., 2011; Medina-Sánchez, 2013; Medlin et al., 2015; Mukherjee et al., 2014; Novakova et al., 2015; Ogrzewalska et al., 2008, 2010, 2011, 2014, 2015; Pacheco et al., 2012; Parola et al., 2007; Ponnusamy et al., 2014; Ramos et al., 2015; Saraiva et al., 2013; Sayler et al., 2014; Schulze et al., 2011; Soares et al., 2015; Stromdahl et al., 2000, 2008; Tarragona et al., 2015; Zhang et al., 2012). For the environmental characterization, we considered a set of 19 bioclimatic layers, derived from temperature and precipitation, obtained from the project Worldclim 1.4 (Hijmans et al., 2005, http://www.worldclim.org) and five topographic layers including elevation, slope, topographic index, northness and eastness aspect taken and derived from de Hydro1k project (Kobelkowsky-Vidrio et al., 2014, http://eros.usgs.gov/#/Find_Data/Products_and_Data_Available/gtopo30/hydro).

To reduce the multidimensionality of the layers, we used a principal component analysis (PCA) using the package ENMGadgets (Barve and Barve, 2014) in R software (R Core Team, 2015). Then, geographic locations were associated with principal component layers and were plotted using the first two components in order to identify the position of the Mexican west coast record.

3. Results

The hard ticks collected correspond to *Amblyomma mixtum* (5 individuals, 3 females and 2 males), and *Amblyomma parvum* (20 individuals, 7 females, 5 males and 8 nymphs). Twenty specimens were analysed (3 [2 females, 1 male] *A. mixtum* and 17 [6 females, 3 males, 8 nymphs] *A. parvum*), only two *A. mixtum* were positive for rickettsia DNA. None of *A. parvum* resulted positive. We detected a single *Rickettsia* haplotype for each rickettsial gene in two *Amblyomma mixtum* ticks (one female and one male) (GenBank accession numbers: KX363842–KX363847), which showed high homology (99–100%) with the *gltA*, *ompA* and *ompB* sequences of "*Ca.* R. amblyommii" available already in GenBank (CP012420, CP003334). Our sequences and those of reference for "*Ca.* R. amblyommii" deposited in GenBank are grouped in a single cluster in three dendrograms generated (Fig. 1A–C).

We generated an occurrence database of 123 unique and published geographical records distributed in eleven countries: Argentina, Belize, Brazil, Colombia, Costa Rica, French Guiana, Honduras, Mexico, Panama, Paraguay and the USA (Fig. 2A).

This new record of "*Ca.* R. amblyommii" in Mexico is located approximately 700 km from the site of a previous unpublished record made in Veracruz, Mexico (Medina-Sánchez, 2013), the only of this species that it is possible locate in the country, given that it exists reference to other possible record in northeastern Mexico but there is no reference to the specific locality (Sosa-Gutierrez et al., 2015). Both georreferenced records in Mexico are located in eastern and western coasts, yet it is noteworthy that our results represent the most western geographic record known for "*Ca.* R. amblyommii" (Fig. 2A).

Considering the environmental characteristics used, it was possible to obtain 60.48% of the cumulative proportion of variance found in the layers in the first two components (PCA1 and PCA2, loading values for each component in Table 1). It is possible to observe that almost all records form a clump considering the environmental characteristics, and the Mexican records share values with records found in Brazil and Costa Rica which are the closest points in the graph (Fig. 2B). However, our new geographic record does not represent a new environmental condition where the species is distributed.

4. Discussion

Here we report for the first time the presence of "*Ca.* R. amblyommii" in *A. mixtum* obtained on the west coast of Mexico. "*Ca.* Rickettsia amblyommii" has previously been referred in 14 species of the *Amblyomma* genus (which represents 10.7% [14/130] of total species described [Guglielmone et al., 2010]) which include: *A. americanum* and *A. maculatum* in the USA (Apperson et al., 2008; Fitak et al., 2014; Fritzen et al., 2011; Gaines et al., 2014; Hermance et al., 2014; Killmaster et al., 2014; Moncayo et al., 2010; Ponnusamy et al., 2014; Sayler et al., 2014; Schulze et al., 2011; Smith et al., 2010; Trout et al., 2010; Zhang et al., 2012); *A. auricularium*, *A. cajennense*, *A. coelebs*, *A. geayi*, *A. humerale*, *A. longirostre*, *A. mixtum*, *A. neummanni*, *A. oblongoguttatum*, *A. ovale*, *A. sculptum* and *A. tonelliae* in Central and South America (Alves et al., 2014;

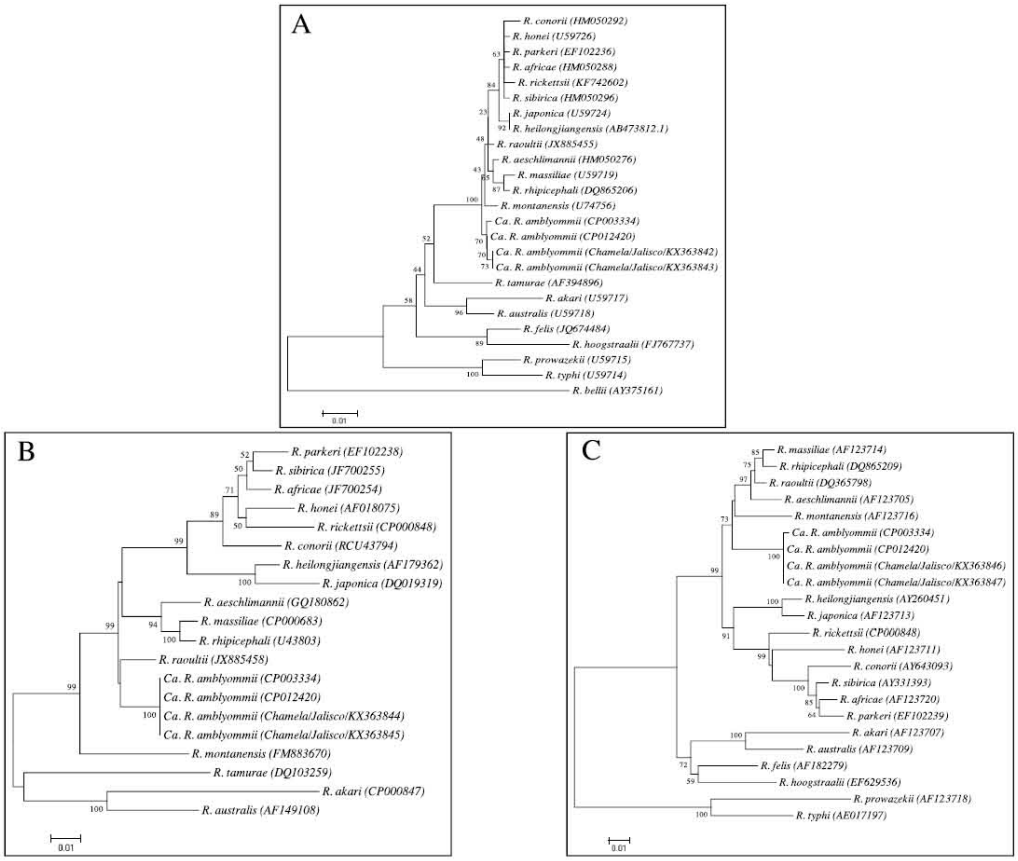

Fig. 1. Neighbor-joining phylogenetic trees generated with partial sequences of the *gltA* (A), *ompA* (B) and *ompB* (C) genes from several members of the genus *Rickettsia* using Kimura two-parameter distance model. Bootstrap values higher than 50 are indicated at the nodes.

Bermúdez et al., 2009, 2011; Hun et al., 2011; Labruna et al., 2004b, 2007; Ogrzewalska et al., 2008, 2010, 2011; Saraiva et al., 2013; Soares et al., 2015; Tarragona et al., 2015).

Nine of these species have been recorded in Mexico (*A. americanum*, *A. auricularium*, *A. coelebs*, *A. humerale*, *A. longirostre*, *A. maculatum*, *A. mixtum*, *A. oblongoguttatum*, *A. ovale*), and only one species has been screened for "*Ca.* R. amblyommii", although in Mexico 26 species of *Amblyomma* have been recorded (Guzmán-Cornejo et al., 2011). Therefore, more tick species must be sampled in order to identify the entire range of arthropods possibly associated with rickettsia species.

This is the most western record of "*Ca.* R. amblyommii" for the Western Hemisphere; however, it does not represent a new environmental record, such as it has happened with other new geographic records for other groups (e.g., Hanna et al., 2016). This is possible because the environmental characteristics in western Mexico (characterized by precipitation, temperature and topographic variables, Rzedowski, 1978) are represented in other parts of the distribution of the species, such as the Caatinga and the Chaco in South America (Sampaio, 1995; Zanella, 2011).

Currently, no hypothesis of the potential distribution of "*Ca.* R. amblyommii" has been proposed, despite that *A. mixtum* has been associated to dry and humid areas in Mesoamerica and the Caribbean (Estrada-Peña et al., 2014). However, it is possible to identify that the environmental characteristics of "*Ca.* R. amblyommii" are not only restricted to tropical areas. Latitudinal extreme records found in the United States, for example, are found in temperate forests (Olson et al., 2001) and represent limits in the environmental distribution considering cold temperatures that are also a limitation for *Amblyomma* ticks (Cabrera and Labruna, 2009; Estrada-Peña et al., 2014).

The close position of both Mexican records considering the environmental characteristics, is due that the similarities of tropical lowlands present in both Mexican coasts (Rzedowski, 1978). It is important to say that a recent report in northeastern Mexico could be associated to tropical lowlands, but we were not able to include it because we could not georreference it (Sosa-Gutierrez et al., 2015). Also, it is necessary to consider that the west coast of Mexico, where the new record was found, is dominated by the tropical deciduous forests which extend along the Pacific coast from northwestern Mexico to northwestern Costa Rica (Olson et al., 2001), where it could be possible to find more records for the species.

For this reason, it is essential to undertake systematic studies covering new areas including the west coast of Mexico and all Central American countries, in order to identify the distribution of "*Ca.* R. amblyommii", considering that *A. mixtum* is the most

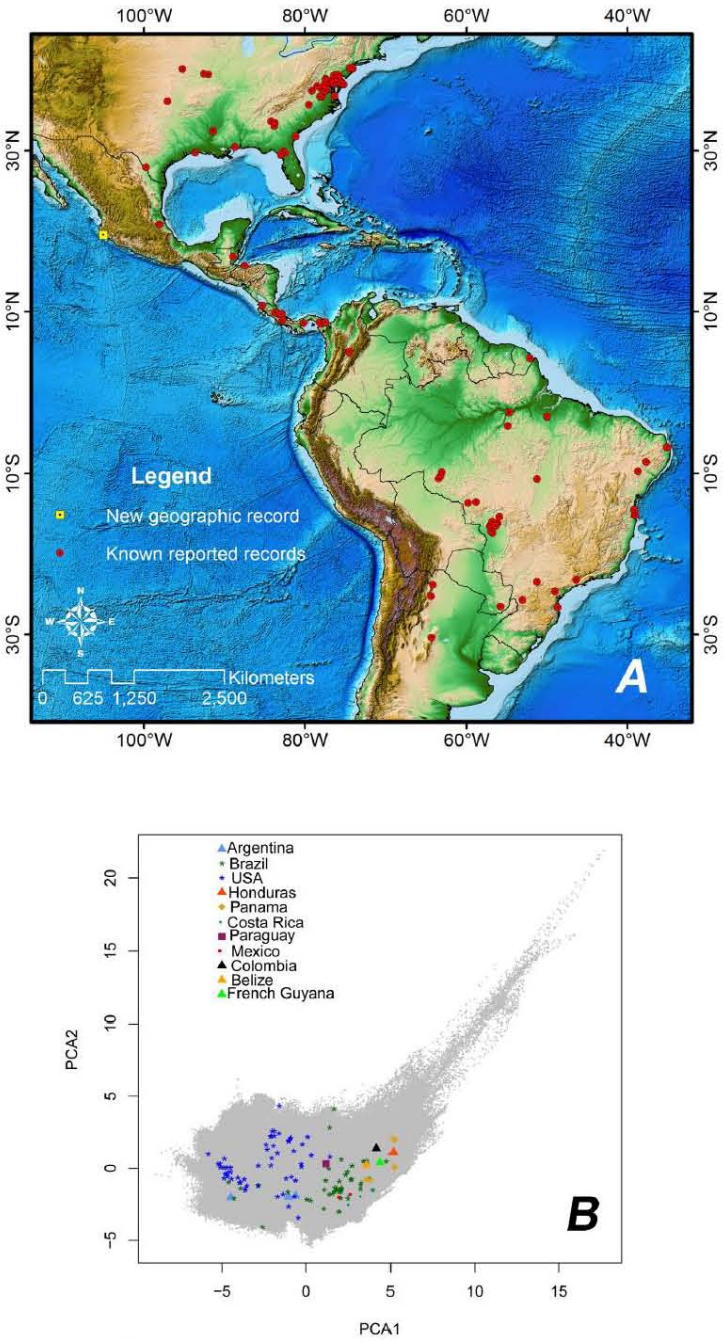

Fig. 2. Distribution of ¨*Candidatus* Rickettsia amblyommii¨. A) Geographic records of "*Ca.* R. amblyommii" in the Western Hemisphere based on literature (see Material and methods). We highlight in a yellow square the new geographic record found in Jalisco on the western coast of Mexico. B) Environmental characteristics of the records with respect to first two principal components (PCA1 and PCA2) derived from 19 bioclimatic and five topographic layers (Table 1). Grey dots represent the environmental characteristics present in the Western Hemisphere. Red circles represent the two georreferenced localities in Mexico. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Table 1
Environmental characteristics and their loading values from the principal component analysis (PCA). PC1 and PC2 refer to the first two principal components of the PCA. We have highlighted in bold the three highest loading values for each principal component.

Original environmental variables	PC1	PC2
Annual mean temperature	**0.2824**	−0.1617
Mean diurnal range	−0.1809	−0.2389
Isothermality	0.2531	−0.1385
Temperature seasonality	−0.2578	0.1574
Maximum temperature of warmest month	0.1577	−0.1765
Minimum temperature of the coldest month	**0.2881**	−0.1344
Temperature annual range	−0.2706	0.0865
Mean temperature of wettest quarter	0.2099	−0.1399
Mean temperature of driest quarter	0.2563	−0.1483
Mean temperature of warmest quarter	0.2202	−0.1180
Mean temperature of coldest quarter	**0.2835**	−0.1679
Annual precipitation	0.2543	0.2339
Precipitation of wettest month	0.2631	0.0793
Precipitation of driest month	0.1231	**0.4276**
Precipitation seasonality	0.0323	**−0.4191**
Precipitation of wettest quarter	0.2624	0.0918
Precipitation of driest quarter	0.1356	**0.4260**
Precipitation of warmest quarter	0.1709	0.2118
Precipitation of coldest quarter	0.1882	0.2368
Altitude	−0.1463	−0.1257
Eastness slope	−0.0109	−0.0256
Northness slope	0.0089	0.0156
Slope	−0.0758	−0.0505
Topographic index	0.0870	0.0493

widely distributed species of *Amblyomma* in the country and the Mesoamerican region (Guzmán-Cornejo et al., 2011; Nava et al., 2014).

Since the pathogenicity of "*Ca.* R. amblyommii" is still unknown, cross-reactivity with other pathogenic members of spotted fever group (especially *Rickettsia rickettsii*) has been reported (Apperson et al., 2008). Additionally, "*Ca.* R. amblyommii"-antibodies have been detected in patients suspected of Rocky Mountain spotted fever, and the tick *A. mixtum* has been associated feeding on people (Guzmán-Cornejo et al., 2011). For the previously reasons, it is important to consider this rickettsia species as a potential risk for the human population. Moreover, previous reports of patients with spotted fever from the state of Jalisco (CENAPRECE, 2015), call for a careful analysis of tick vectors, as well as the involved *Rickettsia* species that are causing human rickettsial diseases (Blanton et al., 2014).

We conclude that further serological and tick surveillance studies need to be implemented in order to identify the degree of exposure of the human population living close to, or visiting areas of where the environmental characteristics of "*Ca.* R. amblyommii" can be found.

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Capítulo 5 First Record of *Bartonella vinsonii* in the Sucking Louse *Hoplopleura hirsuta* Collected from Hispid Cotton Rats, *Sigmodon hispidus*, in Mexico

First Record of *Bartonella vinsonii* in the Sucking Louse *Hoplopleura hirsuta* Collected from Hispid Cotton Rats, *Sigmodon hispidus*, in Mexico

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First Record of *Bartonella vinsonii* in the Sucking Louse *Hoplopleura hirsuta*[1] Collected from Hispid Cotton Rats, *Sigmodon hispidus*, in Mexico

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Abstract. This study determined the presence of *Bartonella* species in sucking lice from hispid cotton rat, *Sigmodon hispidus* Say and Ord, at San Luis Potosí, Mexico. Louse specimens were processed for morphological identification and evaluated for *Bartonella* DNA. A single sucking louse species was found and identified as *Hoplopleura hirsuta* Ferris. DNA was extracted from 20 adults, and a 379-bp fragment of *Bartonella* citrate synthase (*gltA*) gene was amplified. Six lice (30%) were positive for *Bartonella* DNA, and all sequences found belonged to *Bartonella vinsonii* Brenner et al. To our knowledge, this is the first report of a member of *B. vinsonii* complex associated with sucking lice.

Introduction

Bartonella is a genus of alphaproteobacteria whose members colonize mammalian erythrocytes and endothelial cells (Houpikian and Raoult 2001). At least 30 species of *Bartonella* have been described, several of which have been isolated from various rodent species, particularly cricetids in the United States; yet, their role as potential pathogens remains unknown (Kosoy et al. 1997, Houpikian and Raoult 2001, Iralu et al. 2006). One of the rodents is the hispid cotton rat, *Sigmodon hispidus* Say and Ord, whose range extends from the midwestern United States through interior Mexico (Wilson and Reeder 2005). In the United States, the rat is known to be a natural reservoir of several *Bartonella* genotypes (Kosoy et al.

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1997, Houpikian and Raoult 2001, Bai et al. 2007), but few reports have identified arthropod ectoparasites of hispid cotton rat, e.g., fleas (Abbot et al. 2007) that could potentially maintain and transmit the agents. A number of *Bartonella* species have been detected in sucking lice (Durden et al. 2004, Reeves et al. 2006, Morick et al. 2009), particularly the genus *Hoplopleura* (e.g., *Hoplopleura pacifica* Ewing; *Bartonella rattimassiliensis* Gundi et al., *Bartonella phoceenis* Gundi et al. (Reeves et al. 2006); *Hoplopleura sciuricola* Ferris; and *Bartonella washoensis* Regnery et al. (Durden et al. 2004)). However, the role of sucking lice as potential vectors is unclear. *Bartonella*, a pathogen not well studied in Mexico, was previously reported only in sucking louse from homeless people at Mexico City (Alcantara et al. 2009) and in several wild rodent species and their associated fleas from Chihuahua (Rubio et al. 2014, Fernández-González et al. 2016). During investigations of population dynamics of hispid cotton rat at San Luis Potosi, their lice were collected for *Bartonella* detection. Here, we report for the first time DNA of *Bartonella* in sucking lice associated with hispid cotton rat in Mexico.

Materials and Methods

In August 2012, a cross-sectional study determined the presence of *Bartonella* species in sucking lice from hispid cotton rats caught at plantations of sugarcane, *Saccharum officinarum* L., at Obregón (-99.33, 22.51) and Miguel del Naranjo (-99.33, 22.49), State of San Luis Potosí, Mexico, at the southern limit of the geographical distribution of this mammal species (Wilson and Reeder 2005). Sherman traps were used to capture rodents under permit FAUT-0069 from the Secretaría del Medio Ambiente y Recursos Naturaless; hosts were sacrificed in accordance with the Guidelines of the American Society of Mammalogists for the Use of Wild Mammals in Research (Gannon and Sikes 2007). Lice were removed from rodent bodies and fixed in 96% ethanol. Some louse specimens were processed for morphological identification using keys of Kim et al. (1986), and another group was selected for molecular identification of *Bartonella*. DNA from adult sucking lice was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). A 379-bp fragment of the citrate synthase (*gltA*) gene was amplified for *Bartonella* DNA detection using primers BhCS781.p and BhCS1137.n (Norman et al. 1995). The molecular marker has proven to be the most appropriate for specific identification of members of *Bartonella* genus (La Scola et al. 2003). The reaction mixture was 12.5 µl PCR master mix solution (Qiagen), 100 ng of each primer, 6.5 µl nuclease-free water, and 200 ng DNA in a final volume of 25 µl (Sánchez-Montes et al. 2016). PCR conditions were reported previously (Rubio et al. 2014). PCR-positive products were sequenced upstream at Laboratorio de Biología Molecular y de la Salud, Universidad Nacional Autónoma de México, using the same primers as in the initial PCR amplification. The sequences were edited and aligned using Mega 5.01 and compared using Basic Local Alignment Search Tool (BLAST) with references available in GenBank. Additionally, multiple alignments used CLUSTAL W; nucleotide sequence divergences were calculated using the Kimura two-parameter distance model, and a neighbor-joining phylogenetic tree was generated. Based on the results, we identified the *Bartonella* species by the criterion of 97% homology established by La Scola et al. (2003).

Results

In total, six of 51 rodents collected (12%) had one sucking lice positive for *B. vinsonii*. Six sucking lice (four female and two male) of 20 found (30%) tested positive for *B. vinsonii* DNA. Three genetic variants were identified: 1) one from a single *H. hirsuta* female at Miguel del Naranjo (GenBank accession no. KT326174), 2) two sequences of two males from Miguel del Naranjo with an identity of 100% between them (GenBank accession no. KT326175, KT326176), and 3) three sequences of females from Obregon plantation with 99 to 100% sequence identity (GenBank accession no. KT326177-KT326179). All sequences in the study belonged to a single species *B. vinsonii* (96-98% homology), a member of *B. vinsonii* complex according to Kosoy et al. (2012), and previously referred to as phylogenetic group B (Kosoy et al. 1997) (Fig. 1). Our sequences and those deposited in GenBank isolated from hispid cotton rat were grouped into two subgroups, clearly separated from those formed by sequences from *Neotoma albigula* Hartley and *Onychomys* sp. previously identified in Chihuahua, northeastern Mexico (Fig. 1).

Discussion

To our knowledge, this is the first report of *B. vinsonii* associated with sucking lice. Previous studies identified *Bartonella vinsonii vinsonii* Brenner et al. in fleas (*Ctenophthalmus pseudagyrtes* Baker) (Reeves et al. 2007) from *Microtus* sp., but there have been no systematic studies of other ectoparasite groups to know which are potential vectors of the pathogen.

There is much genetic diversity among the samples analyzed, with two groups well defined: one belonged to Obregon plantation and the other to Miguel del Naranjo, even when both locations are close to each other. The presence of multiple genetic variants from nearby localities has been previously reported for isolates of *Bartonella grahamii* (Birtles et al.) in Northern Europe and America (Berglund et al. 2010).

Additionally this record represents the first for the state and farther south to the continent. Because *H. hirsuta* is widespread in six states of Mexico (Sanchez-Montes et al. 2013) and parasitizes several species of sigmodontines (*Sigmodon arizonae* Mearns, *Sigmodon ochrognathus* Bailey, and *Sigmodon toltecus* Saussure) the louse could function as a bridge vector, facilitating the spread of *Bartonella* between rodent species (Durden and Musser 1994, Sanchez-Montes et al. 2013); however, more study is needed. Close surveillance of the bacteria in *H. hirsuta* and its mammal hosts is required because pathogenicity is unknown and the congeneric taxa *Bartonella vinsonii arupensis* Welch et al. and *B. vinsonii berkhoffii* Breitschwerdt et al. can be pathogenic to humans, causing endocarditis (Roux et al. 2000, Fenollar et al. 2005). It is therefore essential to identify the spread of the pathogen in lice and to confirm experimentally its vector competence to understand the ecology of transmission and reduce the impact of this potential zoonotic pathogen to at-risk human populations in Mexico.

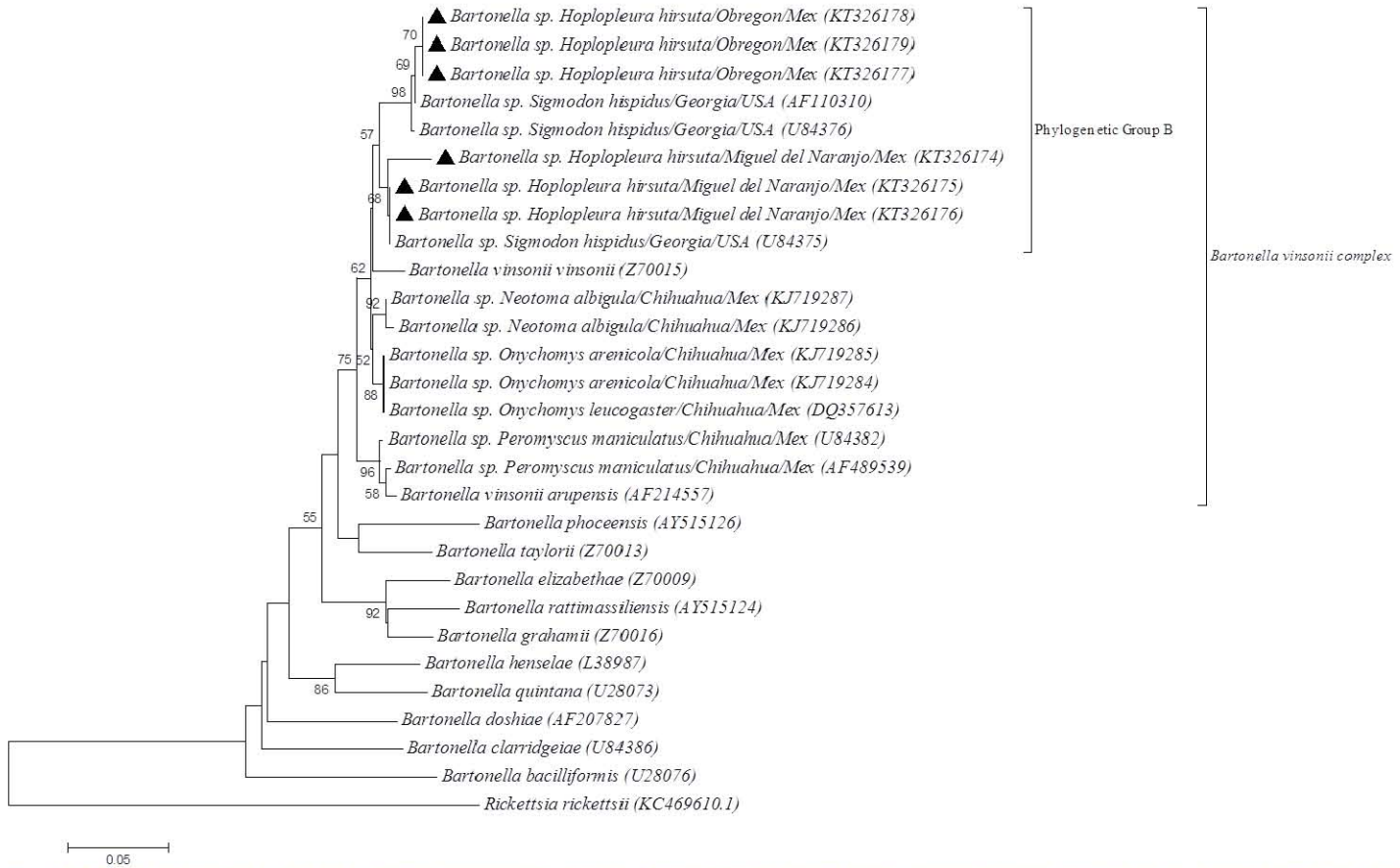

Fig. 1. Neighbor-joining phylogenetic tree generated using Kimura two-parameter distance model with partial sequences of the *gltA* gene from several members of the genus *Bartonella*. Bootstrap values greater than 50 are indicated at the nodes. Sequences in the study are marked with solid triangles.

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Durante el presente trabajo colaboré activamente en proyectos relacionados con la línea de investigacion, los cuales enlisto a continuación:

Espinosa-Martínez DV, Sánchez-Montes S, Ríos-Muñoz CA, Berzunza-Cruz M, Becker I (2015) New wildlife hosts of *Leptospira interrogans* in Campeche, México. *Revista do Instituto de Medicina Tropical de São Paulo*. 57: 181-183.

Sánchez-Montes S, C. Guzmán-Cornejo, Martínez-Nájera, Y, Becker, I, Venzal JM, Labruna MB. (2016) *Rickettsia lusitaniae* associated with *Ornithodoros yumatensis* (Acari: Argasidae) from two caves in Yucatan, Mexico. *Ticks and Tick-Borne Diseases*. 7: 1097-1101.

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BRIEF COMMUNICATION

NEW WILDLIFE HOSTS OF *Leptospira interrogans* IN CAMPECHE, MEXICO

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SUMMARY

Leptospira interrogans has been identified to cause leptospirosis, a widespread zoonotic disease that has been identified in domestic and wild animals. This work analyzed kidneys from two species of wild rodents from the state of Campeche, Mexico. Analyses were made by PCR using specific primers for detection of *Leptospira interrogans* DNA. The rodent species that tested positive were *Heteromys gaumeri* and *Ototylomys phyllotis*, both of which are new hosts for the bacteria in Southeastern Mexico. These records provide new insights into the disease's transmission that should be studied carefully in order to identify other potential host species, including humans, which are at risk of becoming infected if they are in contact with infected wildlife.

KEYWORDS: Wildlife hosts; *Leptospira interrogans*; Campeche; Mexico.

Several species of the genus *Leptospira* cause leptospirosis, a zoonosis of urban distribution[3,6]. Wild and domestic mammals (160 species) have been identified as hosts for these bacteria worldwide[2,6]. *Leptospira interrogans* has mainly been identified in domestic mammals because they have direct contact with humans[4,5,6]. However, in Neotropical areas, such as Panama[21], the Peruvian Amazon[6,8] and the city of São Paulo[16], some wild mammals (bats, carnivores, marsupials and rodents) have been identified as hosts of *L. interrogans*.

In Mexico, records of wildlife hosts for *L. interrogans* are scarce and widely scattered across different states (e.g. *Didelphis virginianus* in Yucatán[25] [Southeastern Mexico], *Odocoileus virginianus* in Coahuila[9] [Northern Mexico] and *Zalophus californianus* in the Gulf of California[1,18]). A study carried out in Cozumel, Quintana Roo, identified a 21.5% seroprevalence of *Oryzomys couesi cozumelae*[24]. In Tamaulipas, Northeastern Mexico, five species of wild rodents (*Baiomys musculus*, *Liomys irroratus*, *Oryzomys alfaroi*, *Peromyscus leucopus* and *Sigmodon hispidus*) tested positive for different serovars of *L. interrogans* by Microscopic Agglutination Technique (MAT)[22]. However, there are no records of wildlife hosts reported in Campeche, and in the Yucatan Peninsula only one species of rodent has been previously reported[24]. For this reason, the aim of this paper is to report two new species of wild rodents that are hosts of *L. interrogans* in Calakmul, Campeche, Mexico.

Ten rodents were collected (collection permit FAUT-0170) on August 17th, 2013 from the Yaax'che camp, Calakmul, Campeche, Mexico (located 43 km SSE from the archeological zone of Calakmul, 18° 29' 14'' N, 89° 53' 57'' W). These specimens were killed in compliance with the guidelines of the American Society of Mammalogy for the Use of Wildlife Mammals in Research[17]. All specimens were identified and deposited at the Museo de Zoología "Alfonso L. Herrera" in the Facultad de Ciencias (MZFC) of the Universidad Nacional Autónoma de México.

For the identification of *Leptospira* DNA in these rodents, one kidney was aseptically collected and deposited in 70% ethanol. A portion of 25 mg of kidney tissue was processed for DNA extraction using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's specifications (using the Purification of Total DNA from Animal Tissues Protocol). After extractions were done, a multiplex PCR was performed using primer sets G1/G2 (specific for the detection of pathogenic leptospires) and B64I/B64II (specific for *Leptospira kirschneri*) with expected products of 285 bp and 563 bp, respectively[19]. Additionally, the positive samples were analyzed using specific primers for the identification of pathogenic leptospira species[23]. The reaction mixture consisted of 12.5 µL of GoTaq® Green Master Mix, 2X of Promega Corporation (Madison, WI, USA), using a pair of primers, Intergroup A fwd and Intergroup A rev (100 ng each), 6.5 µL nuclease-free water and 200 ng DNA in a final volume of 25 µL.

In order to minimize cross-contamination and to avoid false positive results, a negative control (i.e. reaction mix without DNA) and a positive

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control (i.e. reaction mix and *L. interrogans* serovar Pomona DNA) were both included. Each PCR reaction was performed in triplicate.

The PCR products were analyzed by electrophoresis on 1.5% agarose gels, using a 100 bp molecular weight marker (Nucleic Acid Markers, LMW DNA Ladder of BioLabs) in 1X TAE buffer. Gels were stained with SYTO® 60 nucleic acid stain (Invitrogen by Life Technologies CA, USA) and visualized using an ODYSSEY CLx Imaging System (LICOR Biosciences).

Two rodents collected at Yaax'che camp, Calakmul, Campeche, Mexico that tested positive using the G1/G2 primers were identified as *Heteromys gaumeri* (temporary catalog RAVGA014) and *Ototylomys phyllotis* (temporary catalog RAVGA013).

These tests were confirmed positive with primers of Intergroup A designed by REITSTETTER[23], which specifically amplify a segment of 396 bp of *L. interrogans* DNA. *Leptospira kischneri* was not detected in any of the samples analyzed, and the DNA of *L. interrogans* was not found in any of the negative controls (Fig. 1).

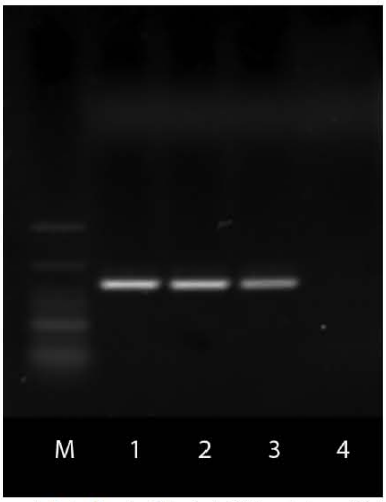

Fig. 1 - Agarose-gel electrophoresis of the single PCR products amplified with primers of Intergroup A designed by REITSTETTER[23]. M: 100 bp DNA marker ladder; Lane 1: *Heteromys gaumeri* (RAVGA014); Lane 2: *Ototylomys phyllotis* (RAVGA013); PCR Controls; Lane 3: Positive control (396 bp, *L. interrogans* serovar Pomona DNA); Lane 4: Negative control (without DNA).

Climate affects the timing and intensity of outbreaks of infectious diseases[14,15]. It has been stated by several authors[3,6,20,26] that adverse climatic events, such as hurricanes and floods, are related to the timing and intensity of *Leptospira* outbreaks. In the case of the present study, the presence of two tropical storms that occurred before and after the specimen's collection[12,13], allowed for speculation regarding the study's findings of *L. interrogans*.

This is the first work that identifies *Heteromys gaumeri* and *Ototylomys phyllotis* as new hosts for *L. interrogans*, by using the set of primers designed by REITSTETTER[23] to identify pathological samples. Moreover, the study area in which the specimens were collected corresponds to a new locality in Mexico, where the presence of the bacteria had not been previously reported. The presence of *L. interrogans* in wild rodents from the same locality should be studied carefully in order to identify the possibility of other species and particularly humans of this area being infected. The author's suggestion is based on previous studies made on domestic animals and humans. In the case of domestic animals (bovines, pigs and dogs) a study revealed a general positivity of 30.5%[10], while a more recent study showed a general positivity of 21.3% registered in dogs of Campeche city[7]. Particularly in the case of human leptospirosis, incidence varied from 0.7-2.2/100,000 inhabitants, with a general seroprevalence of 14.2%[11,25].

Since extreme weather events have been reported to promote the presence of *Leptospira* outbreaks[6], it is essential to further analyze potential reservoirs of several pathogenic species of *Leptospira* in order to identify the dynamics of the transmission between wild mammals and peri-urban human populations, in order to reduce the risks of a potential leptospirosis outbreak in vulnerable groups such as biologists, national and foreign campers and tourists that visit the study area.

RESUMEN

Nuevos huéspedes silvestres de *Leptospira interrogans* en Campeche, México

Lepstospira interrogans ha sido identificada como uno de los agentes causantes de la leptospirosis, una zoonosis ampliamente distribuida, la cual se ha identificado en numerosos animales domésticos y silvestres. En este trabajo se analizaron los riñones de dos especies de roedores silvestres procedentes del estado de Campeche, México mediante la técnica de PCR con iniciadores específicos para la detección de DNA de *Leptospira interrogans*. Las especies de roedores que resultaron positivas corresponden a *Heteromys gaumeri* y *Ototylomys phyllotis*, ambas representan nuevos registros de huéspedes para la bacteria en el sureste de México. Estos nuevos huéspedes deberán ser estudiados cuidadosamente con el fin de determinar la posibilidad de que otras especies de animales, y en particular los humanos, entren en contacto con el patógeno presente en animales silvestres.

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Rickettsia lusitaniae associated with *Ornithodoros yumatensis* (Acari: Argasidae) from two caves in Yucatan, Mexico

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Mexico

ABSTRACT

The genus *Rickettsia* includes obligate intracellular bacteria transmitted by several hematophagous arthropods such as ticks, fleas and sucking lice. In particular hard ticks (Ixodidae) have been cited as the main vectors of pathogenic rickettsiae in Mexico. However, there have been only two records of a single *Rickettsia* species associated with Mexican soft ticks (Argasidae). In this study, we searched for rickettsial DNA in argasid ticks (13 adults and eight nymphs of *Ornithodoros yumatensis*) from two bat caves in the state of Yucatan, Mexico. Additionally one larva collected in a cave from Chiapas, Mexico, and associated with *Desmodus rotundus* was used to corroborate the tick taxonomic determination. Of these, nine ticks (43%) yielded expected PCR products for the rickettsial *gltA* gene. These PCR-positive ticks were tested with additional PCR protocols targeting the rickettsial genes *gltA*, *ompA* and *ompB*. DNA partial sequences from these genes showed 99–100% identities with *Rickettsia lusitaniae*, an agent isolated from *O. erraticus* in Portugal, and closely related to *R. felis* and *R. hoogstraalii*. Based on the results from this study, the inventory of rickettsiae distributed in Mexico increases from six to seven species.

1. Introduction

The genus *Rickettsia* includes Gram-negative obligate intracellular bacteria that are associated with hematophagous arthropods such as ticks, fleas and lice, and with non-hematophagous insects, such as leeches, hydras, and amoebas (Dykova et al., 2003; Murray et al., 2016). While most of the reports of rickettsiae have been from ticks of the family Ixodidae (hard ticks) (Parola et al., 2013). A relatively low number of studies have been conducted on ticks of the Argasidae family (soft ticks), mainly from Europe, Asia, Africa or North America (Table 1). In Mexico, only six species of *Rickettsia* have been reported (Labruna et al., 2011; Sosa-Gutierrez et al., 2015). To the best of our knowledge, only *R. rickettsii* was referred to be in association with an argasid (Table 1).

The number of ticks of the family Argasidae in Mexico includes 32 species (Pérez-Ortiz et al., 2014) and most of them have been poorly studied, specifically those inhabiting caves. *Ornithodoros yumatensis* (Cooley and Kohls, 1941) is a species associated with bats and caves and it was described from Crystal cave, which is 10 miles southeast of Winkelman, Arizona. Its current geographical distribution includes the USA, Colombia, Mexico, Nicaragua and Venezuela (Guglielmone et al., 2003). This species has been recorded in association with Molossidae, Phyllostomidae and Vespertilionidae bats (Cooley and Kohls, 1941; Kohls et al., 1965; Jones et al., 1972; Dooley et al., 1976). However, little is known of its biology, ecology and associated microorganisms.

Our current work is part of a project aimed at exploring the diversity of argasid ticks in Mexican caves and identifying the rickettsiae associated with these ticks.

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Table 1
Records of species of the genus *Rickettsia* associated with Argasidae.

Rickettsia species	Tick	Country	Reference
R. bellii	*Argas cooleyi*	USA, America	Philip et al. (1983)
	Ornithodoros concanensis	USA, America	Philip et al. (1983)
R. bellii-like	*Carios capensis*	Western Indian Ocean Islands	Dietrich et al. (2014)
R. felis-like	*Carios capensis*	USA, America	Reeves et al. (2006)
R. hoogstraalii	*Argas persicus*	Ethiopia, Africa	Pader et al. (2012)
	Carios capensis	Japan, Asia	Kawabata et al. (2006)
	Carios capensis	USA, America	Mattila et al. (2007)
	Carios capensis	USA, America	Duh et al. (2010)
	Carios sawaii	Japan, Asia	Kawabata et al. (2006)
R. hoogstraalii-like	*Carios capensis*	Western Indian Ocean Islands	Dietrich et al. (2014)
R. lusitaniae	*Ornithodoros erraticus*	Portugal, Europe	Milhano et al. (2014)
R. rickettsii	*Ornithodoros nicollei*	Mexico, America	Silvia-Goytia and Elizondo (1952)
	Otobius lagophilus	Mexico, America	Silvia-Goytia and Elizondo (1952)
R. slovaca-like	*Argas persicus*	Armenia, Asia	Rehácek et al. (1977)
Rickettsia spp.	*Argas dewae*	USA, America	Parola et al. (2013)
	Argas vespertilionis	France, Europe	Socolovschi et al. (2012)
	Carios capensis	Algeria, Africa	Lafri et al. (2015)
	Carios kelleyi	USA, America	Loftis et al. (2005)
	Ornithodoros erraticus	Algeria, Africa	Lafri et al. (2015)
	Ornithodoros moubata	Tanzania, Africa	Cutler et al. (2006)
	Ornithodoros rupestris	Algeria, Africa	Lafri et al. (2015)

1.1. Material and methods

Tick specimens were collected during October 2014 in two caves from the state of Yucatan, Mexico: Chocantes cave, Tekax, 20°12′10″N, 89°17′58″W, temperature of 27 °C, and 91% relative humidity (RH); and a cave near cenote Bal-mil, Homún, 20°45′10″N, 89°14′65″, temperature of 27 °C, and 98% RH. Ticks were collected from crevices in the walls of the caves, and fixed with 96% ethanol.

The ticks were morphologically identified following published descriptions and taxonomic keys (Cooley and Kohls, 1941, 1944). To confirm conspecific identification of nymphs and adults, the DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) from one nymph, and a female collected in the Chocantes cave, a male tick from a cave near cenote Bal-mil. An additional larva was used to corroborate the taxonomic determination. It was collected from Naranjo cave, Cintalapa, Chiapas (16°51′6.80″N, 93°42′16.30″W, temperature of 24 °C, and 100% RH), ex *Desmodus rotundus*. The ticks were processed by a PCR protocol targeting a partial segment (approximately 400 bp) of the mitochondrial 16S rRNA gene of ticks, as reported elsewhere (Norris et al., 1996).

Posteriorly, the DNA extracted from the ticks (using the same DNA extraction protocol mentioned above) was screened for rickettsial infection by PCR. First, the tick DNA samples were tested by a PCR protocol targeting a 381 bp fragment of the citrate synthase (*gltA*) of *Rickettsia* spp. (Regnery et al., 1991). Positive samples were further tested by three other PCR protocols: one targeting an 800 bp of the rickettsial *gltA* gene (de Sousa et al., 2006), one targeting an 862 bp fragment of the rickettsial 135-kDa outer membrane protein gene (*ompB*) (Roux and Raoult, 2000), and one targeting a 532 bp fragment of the rickettsial 190-kDa outer membrane protein gene (*ompA*) (Regnery et al., 1991). Negative (a reaction mix without DNA template) and positive (*R. rickettsii* DNA) controls were included. The PCR products were resolved in 2% agarose gels and visualized in an ODYSSEY CLx Imaging System (LI-COR Biosciences). The PCR products of the expected size were DNA sequenced at Laboratorio de Biología Molecular y de la Salud, Universidad Nacional Autónoma de México. The sequences generated were compared to those deposited in GenBank using the BLAST tool (http://www.ncbi.nlm.nih.gov).

Partial sequences of the three rickettsial genes (*gltA*, *ompB*, *ompA*) were concatenated and aligned with corresponding sequences of *Rickettsia* species available in GenBank (Table 2) using the Clustal W algorithm of MEGA 6.0 software. The nucleotide substitution model was the Tamura three parameter model (T92) with discrete Gamma distribution (+G) based on the lowest BIC (Bayesian Information Criterion) score (5157.26). A phylogenetic analysis was performed using the Maximum Likelihood method (ML) and topology support was tested by bootstrap over 1000 replications.

2. Results

A total of 21 ticks were collected and identified as 13 adults (seven females and six males) and eight nymphs of *O. yumatensis*. Thirteen (five females, two males, and six nymphs) of these ticks were collected at Chocantes cave, whereas the remaining eight (two females, four males, and two nymph) were collected in the cave near cenote Bal-mil.

Fragments of approximately 400 bp of the gene 16S rRNA from those analyzed specimens [larva (GenBank accession number: KX668415), nymph (GenBank accession number: KX668414), female (GenBank accession number: KX668413) and male (GenBank accession number: KX668412) were 98.1–99.2% identical to each other, which confirmed that they most likely represent the same species. Using the Blast analysis, the sequences of *O. yumatensis* were, at most, similar to the *Ornithodoros microlophi* (87%) sequence available in GenBank (JX455899), and when comparing the similarity with the species in which *R. lusitaniae* was originally described (*O. erraticus* GenBank KC311541), the similarity was 79%.

All 21 ticks were tested individually by PCR for *Rickettsia* DNA. Of these, nine ticks (43%) (two females and three males from cave near cenote Bal-mil and three females, one nymph from Chocantes cave) yielded expected PCR products for the fragment of 381 bp of the *gltA* gene. To identify the rickettsial species, positive samples were additionally tested for *gltA*, *ompA* and *ompB* genes. The sequences obtained from the nine ticks were 100% identical to each other for each corresponding gene, which were deposited in GenBank under the following accession numbers: KX377431, KX377432, and KX377433 for the *gltA*, *ompA*, and *ompB* genes, respectively. Using BLAST analysis, these sequences were 100% (654/654 bp), 99.6% (455/457 bp), and 98.9% (771/779 bp) identical to corresponding sequences of the *gltA* (JQ771933), *ompA* (JQ771935), and *ompB* (JQ771937), respectively, of *R. lusitaniae*.

Table 2
Rickettsia sequences from Genbank used for phylogenetic analysis.

Rickettsia species	*gltA*	*ompB*	*ompA*
Rickettsia bellii	CP000087	–	–
Rickettsia prowazekii	AJ235273	AF123718	–
Rickettsia typhi	AE017197	AE017197	–
Rickettsia aeschlimannii	HM050276	AF123705	GQ180862
Rickettsia africae	U59733	AF123720	JF700254
Rickettsia akari	CP000847	CP000847	CP000847
Rickettsia amblyommii	NC_017028	NC_017028	NC_017028
Rickettsia australis	U59718	AF123709	AF149108
Rickettsia conorii	AE006914	AY643093	RCU43794
Rickettsia felis	CP000053	CP000053	–
Rickettsia lusitaniae	JQ771933	JQ771937	JQ771935
Rickettsia japonica	U59724	AF123713	DQ019319
Rickettsia heilongjiangensis	AB473812	AY260451	AF179362
Rickettsia honei	AF022817	AF123711	AF018075
Rickettsia hoogstraalii	FJ767737	EF629536	–
Rickettsia massiliae	U59719	AF123714	CP000683
Rickettsia montanensis	NC_017043	AF123716	FM883670
Rickettsia parkeri	EF102236	EF102239	EF102238
Rickettsia philipii	CP003308	CP003308	FJ666087
Rickettsia rhipicephali	DQ865206	DQ865209	U43803
Rickettsia rickettsii	CP000848	CP000848	CP000848
Rickettsia raoultii	JX885455	DQ365798	JX885458
Rickettsia sibirica	U59734	AY331393	JF700255

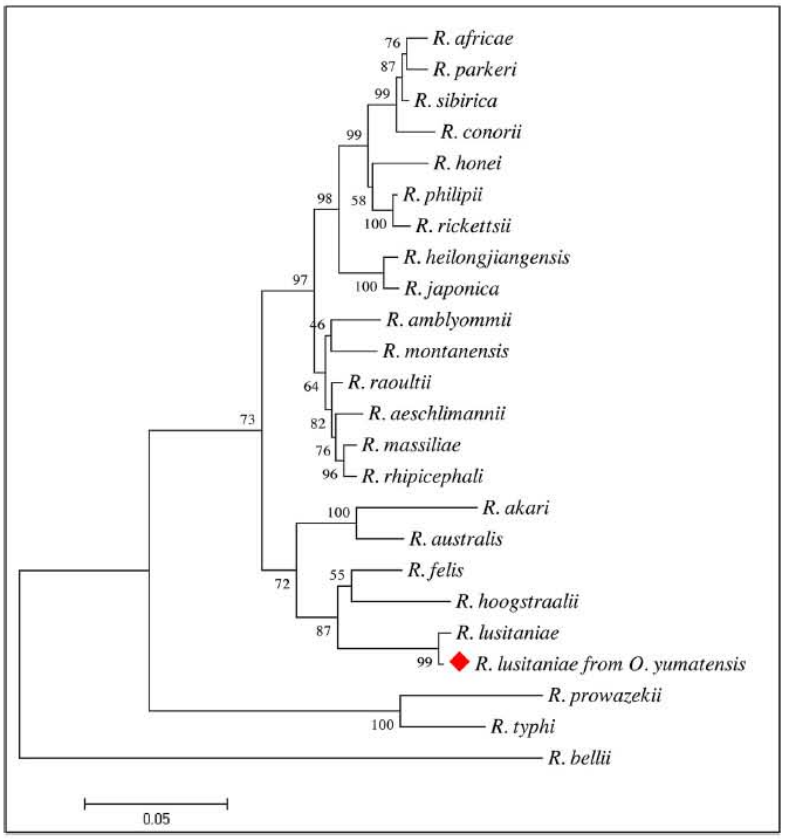

Fig. 1. Maximum likelihood (ML) phylogenetic tree generated with a total of 1840 bp of the *gltA*, *ompA* and *ompB* genes concatenated from members of the genus *Rickettsia*. Bootstrap values higher than 50 are indicated at the nodes.

Due to the variable sizes of the gene fragments of the recognized *Rickettsia* species deposited in GenBank, our phylogenetic analyses included fragments of 648, 432 and 760 bp of the *gltA*, *ompA* and *ompB* genes, respectively, in a concatenated alignment of 1840 bp. The Maximum Likelihood analysis confirms that the *O. yumatensis* rickettsia corresponds to *R. lusitaniae*, a transitional rickettsia group closely related to *R. felis* and *R. hoogstraalii* (Fig. 1).

3. Discussion

It has been proposed that to classify a new *Rickettsia* species, the novel rickettsial agent should not exhibit more than one of the following degrees of nucleotide similarity with the most homologous validated species: ≥99.8, ≥99.9%, ≥98.8, ≥99.2, and ≥99.3% for the 16S rRNA, *gltA*, *ompA*, *ompB* genes, and gene D, respectively (Fournier et al., 2003; Fournier and Raoult, 2009). Because the *O. yumatensis* rickettsia exhibited at least two of the above nucleotide similarity values (100% for *gltA* and 99.6% for the *ompA*) with *R. lusitaniae*, we determined (at this moment) that *O. yumatensis* rickettsia belongs to the *R. lusitaniae* species. Our phylogenetic analysis supports this classification.

We report for the first time the presence of *R. lusitaniae* in America, which was previously reported from Portugal infecting the argasid tick *O. erraticus* (Milhano et al., 2014). We found *R. lusitaniae* associated with *O. yumatensis* ticks in two caves of southern Mexico. Because the geographical distribution of *O. yumatensis* includes the Nearctic and Neotropical regions (Guglielmone et al., 2003), it is possible that *R. lusitaniae* is also present in other countries.

The tick *O. yumatensis* has been associated with bats and caves (Cooley and Kohls, 1941, 1944; Kohls et al., 1965). Due to the limited contact of people with ticks that inhabit caves, the importance of this rickettsia to human health is still unknown. However, bat-associated ticks, including cave-inhabiting species, are also known to bite humans, which usually develop marked local skin reactions following tick bites (Vargas, 1984; Gill et al., 2004; Labruna and Venzal, 2009; Labruna et al., 2014). Further studies are needed to verify if *R. lusitaniae* is tick-transmittable and if it is pathogenic to humans or animals.

The study of argasids in Mexico is still far from being completed and much work is still needed in aspects related to tick biology, ecology, biogeography, systematics, and tick-borne pathogens. With the results of the present study, the inventory of rickettsiae distributed in Mexico increases from six (Labruna et al., 2011; Sosa-Gutierrez et al., 2015) to seven species.

Competing interests

The authors declare they have no competing interests.

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CONCLUSIONES

Este estudio generó nuevos conocimientos sobre la prevalencia de *Bartonella, Rickettsia y Leptospira* y su asociación con roedores y ectoparásitos en México:

1.- Se registraron nuevas asociaciones patógeno-vector en el caso de *Bartonella vinsonii* y las especies de piojo (*Hoplopleura hirsuta*) y pulgas (*Peromyscopsylla hesperomys* y *Plusaetis mathesoni*).

2.- Se registra por primera vez una especie de *Bartonella* asociada con el ratón de Orizaba (*Peromyscus beatae*) en América.

3.- Se registra por primera vez al agente etiológico de la neuroretinitis y endocarditis (*B. elizabethae*) para México.

4.- Todos las localidades representan nuevos registros para la detección de *Bartonella* y *Rickettsia* en México.

5.- Se identificó que la leptospirosis humana se encuentra ampliamente distribuida en México, registrándose en 27 estados y concentrando la mayoría de los casos durante la temporada de lluvias en focos del Centro, Oeste y Sureste del país.

6.- El estado de Hidalgo presentó la mayor diversidad de patógenos zoonóticos regirstrados en el presente trabajo

7.- Se implicó por primera vez a la rata noruega (*Rattus norvergicus*) como potencial reservorio de *Rickettsia typhi* en el estado de Hidalgo.

8.- Se registra por primera vez a *Rickettsia amblyommi* en garrapatas colectadas de personas de la Costa Oeste de México.

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