KARLA ANDRADE DE OLIVEIRA

Amblyomma imitator: A NOVEL VECTOR FOR *Rickettsia rickettsii* IN MEXICO AND SOUTH TEXAS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Agrícola, para a obtenção do título de *Doctor Scientiae*

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Andréa de Oliveira Barros Ribon	**Márcio Antônio Moreira Galvão** (Co-Orientador)
David Hughes Walker	**Donald Hugh Bouyer**

Cláudio Lísias Mafra de Siqueira

(Orientador)

To the Lord, my help and shield (Ps. 33:20),

I dedicate.

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RESUMO

OLIVEIRA, Karla Andrade, D.Sc., Universidade Federal de Viçosa, Fevereiro de 2010. ***Amblyomma imitator*: um novo vetor para *Rickettsia rickettsii* no México e Sul do Texas.** Orientador: Cláudio Lísias Mafra de Siqueira. Co-orientadores: Márcia Rogéria de Almeida Lamêgo, Elizabeth Batista Pacheco Fontes, Juliana Lopes Rangel Fietto e Márcio Antônio Moreira Galvão.

Rickettsioses, doenças de importância mundial, são causadas por bactérias pequenas, gram-negativas e intracelulares obrigatórias, que são transmitidas ao homem através de carrapatos, pulgas, ácaros e piolhos. O gênero *Rickettsia* tem sido classicamente dividido em dois grupos, o grupo tifo (TG) e o grupo de febre maculosa (SFG). Nos Estados Unidos e México, a transmissão da *Rocky Mountain spotted fever* (RMSF) foi atribuída aos carrapatos do gênero *Dermacentor* (*D. variabilis* e *D. andersoni*), *Rhipicephalus sanguineus* e *Amblyomma* (*A. cajennense*). *Amblyomma imitator* possui alta similaridade com *Amblyomma cajennense* e a princípio foi erroneamente identificado como esta espécie de carrapato. A sua distribuição geográfica compreende o sul do Texas (EUA), México e América Central. Neste estudo, uma colônia de carrapatos *A. imitator* coletados no ambiente no Estado de Nuevo Leon, México, em 2007 foi mantida em laboratório em coelhos *naive,* livres de patógenos. Real-time PCR de DNA extraído de ovos postos pela primeira geração de fêmeas mantidas em laboratório, foi realizado utilizando-se os *primers* específicos para *Rickettsia* CS5A e CS6 para a amplificação de um fragmento de 150bp do gene *gltA*. Esta análise indicou a presença de DNA de Rickettsia em algumas das massas de ovos. O organismo foi isolado das massas de ovos em células Vero utilizando-se "Shell vials". Os isolados foram detectados em células Vero por Diff-Quik e Imunofluorescência Indireta utilizando-se anticorpos policlonais de coelho contra *R. rickettsii* cepa *Sheila Smith* (diluído 1:200) e anti-IgG de coelho marcado com isotiocianato de fluoresceína (diluído 1:300). Os isolados foram identificados genotipicamente por seqüenciamento de fragmentos dos genes específicos de *Rickettsia htrA*, *ompB* e *ompA* amplificados a partir do DNA extraído das células infectadas. *Nested*-PCR foi realizada utilizando-se os *primers* 17K3 e 17K5 para a primeira reação e 17KD1 e 17kD2 para a segunda reação para a amplificação de fragmentos de 547 pb e 434 pb, respectivamente, do gene *htrA*.

Para amplificação de um fragmento de 856 pb do gene *ompB* o par de *primers* 120-M59 e 120-807 foi usado. Um fragmento de 533 pb do gene *ompA* foi amplificado com os *primers* Rr190.70F e Rr190.602R. Para uma das amostras um semi-*nested* foi necessário para amplificar um fragmento de *ompA*. Os *primers* Rr190.70F e Rr190.701R foram então utilizados para a primeira reação, e *primers* Rr190.70F e Rr190.602R para a segunda. Análise das seqüências dos fragmentos dos genes *htrA*, *ompA* e *ompB* revelou *R. rickettsii* com identidades de 99%, 100% e 100%, respectivamente. A análise ultraestrutural de tecidos de carrapatos adultos mostrou a presença do agente rickettsial no intestino. Este é o primeiro relato da presença e da transmissão transovariana de *R. rickettsii* por *A. imitator* naturalmente infectado e sugere um papel para esta espécie de carrapato na transmissão de febre maculosa no México e sul do Texas (EUA).

ABSTRACT

OLIVEIRA, Karla Andrade, D.Sc., Universidade Federal de Viçosa, February, 2010. ***Amblyomma imitator:* a novel vector for *Rickettsia rickettsii* in Mexico and South Texas.** Adviser: Cláudio Lísias Mafra de Siqueira. Co- advisers: Márcia Rogéria de Almeida Lamêgo, Elizabeth Batista Pacheco Fontes, Juliana Lopes Rangel Fietto and Márcio Antônio Moreira Galvão.

Rickettsioses, diseases of worldwide importance, are caused by small, gram-negative, obligately intracellular bacteria that are transmitted to humans via ticks, fleas, mites or lice. The genus *Rickettsia* has been classically divided into two groups, the typhus group (TG) and the spotted fever group (SFG). In the United States and Mexico, transmission of Rocky Mountain spotted fever (RMSF) has been attributed to ticks of the genera *Dermacentor* (*D. variabilis* and *D. andersoni*), *Rhipicephalus* (*R. sanguineus*), and *Amblyomma* (*A. cajennense*). *Amblyomma imitator* has close affinity with *Amblyomma cajennense* and was formerly confused with this species. Its distributional range extends from southern Texas (USA) southward through Mexico into Central America (Keirans and Durden, 1998). In this study, a colony of *A. imitator* collected from the field in Nuevo Leon State, Mexico in 2007 was maintained in laboratory on naïve pathogen-free rabbits. Real-time PCR analysis of eggs laid from the first generation of laboratory-reared females using *Rickettsia*-specific primers CS5A and CS6 was carried out for amplification of a fragment of 150bp of the *gltA* gene. This analysis indicated the presence of rickettsial DNA in some of the egg masses. The organism was isolated from the egg masses in Vero cells using shell vials. The bacteria was detected in Vero cells by Diff-Quik staining and indirect immunofluorescence assay using rabbit polyclonal antibody against *R. rickettsii* Sheila Smith strain (diluted 1:200) and fluorescein isothiocyanate-labeled goat anti-rabbit IgG (diluted 1:300). The isolates were genotypically identified by sequencing partial sequences of the *Rickettsia*- specific genes *htrA, ompB*, and o*mpA* amplified from DNA extracted from infected cells. Nested-PCR was performed using primers 17K3 and 17K5 for the first reaction and 17KD1 and 17kD2 for the second reaction for amplification of fragments of 547 bp and 434 bp, respectively, of the *htrA* gene. For amplification of a fragment of 856 bp of the *ompB* gene, the pair of primers 120-M59 and 120-807 was used. A 533 bp fragment of the

ompA gene was amplified using the primers Rr190.70F and Rr190.602R. For one of the samples a semi-nested PCR was necessary to amplify an *ompA* fragment. The primers Rr190.70F and Rr190.701R were used for the first reaction, and primers Rr190.70F and Rr190.602R for the second reaction. Analysis of the sequences of the fragments of *htrA, ompA* and *ompB* genes revealed them to be *R. rickettsii* with identities 99%, 100% and 100%, respectively. Ultrastructural analysis of tissues of adult ticks showed the presence of the rickettsial agent in the tick midgut. This is the first report of the presence and transovarial transmission of *R. rickettsii* by naturally infected *A. imitator* ticks and suggests a role for this species of tick in transmission of Rocky Mountain spotted fever in Mexico and South Texas (USA).

INTRODUCTION

The pathogen

Rickettsia rickettsii is a fastidious, small (0.2–0.5 μm by 0.3–2.0 μm), pleomorphic Gram-negative coccobacillus. Obligately intracellular, the bacteria is not surrounded by a host cell membrane and can grow in the cytoplasm and in the nucleus of infected cells of both arthropod and vertebrate hosts (Burgdorfer et al., 1968).

Electron microscopic study of the *R. rickettsii* outer envelope revealed a trilaminar cell wall (TCW) with an inner leaflet measuring 6.2 to 7.7 nm, an outer leaflet measuring about 2.5 nm, and a "clear" space between the inner and outer leaflets measuring 2.8 to 4.4 nm (Silverman and Wisseman,1978). The cell-wall composition and lipopolysaccharide of the pathogen resemble that seen in other Gram-negative bacteria (Obijeski et al., 1974; Pedersen and Walters, 1978; Anacker et al., 1985). Hayes and Burgdorfer (1982) described the appearance of *R. rickettsii* in optimal physiological stability in ticks that are actively feeding providing essential nutrients, metabolites and temperature required for rickettsial growth. In those conditions *R. rickettsii* shows a prominent “halo”, slime layer (SL) (Silverman et al, 1978), adjacent to the TCW and a slightly electron-dense microcapsular layer (MCL) between the SL and the TCW, approximately 16 nm thick composed of beadlike subunits with a periodicity of approximately 10 nm. Internal to the TCW is a narrow periplasmic space which contains some material of low electron density and that is bordered internally by a plasma membrane. Silverman et al. (1978) suggested that the slime material of Rickettsia is polysaccharide in nature and postulated an antiphagocytic property for the SL also suggesting a role for it in attachment to the host cell in preparation for penetration. Other studies using electron microscopy and ruthenium red staining techniques (Luft 1964; Luft 1971) established the acid protein and polysaccharidic nature of the MCL and of the SL. Hayes and Burgdorfer suggested the importance of these structures in pathogenesis and interactions between *R. rickettsii* and cells of its arthropod and vertebrate hosts. In this study, the authors reported changes in ultrastructure of *R. rickettsii* under growth-limiting conditions infecting starved ticks. The discrete beadlike MCL structures (present under physiologically optimal conditions) dramatically change and resemble stringy strands whose proximal portion appears to fuse with the electron-

dense outer leaflet of the TCW. Besides that the sharp demarcation between the rickettsial SL and the host cell cytoplasm disappear. Supported by examples of a loss or modification of surface components leading to a loss or modification of virulence and pathogenesis of other bacteria found in literature, Hayes and Burgdorfer (1982) suggested that the changes observed in their study are structural changes involved in reactivation, i.e., changes in the pathogenic and virulent nature of *R. rickettsii*.

R. rickettsii possesses two major antigenic surface proteins: outer membrane protein A (OmpA) and outer membrane protein B (OmpB). OmpB is the most abundant surface protein of rickettsiae. Studies suggested that OmpA and OmpB contribute to the adherence and invasion of the host cell (Li and Walker, 1998; Uchiyama, 2006). Uchiyama et al. (2006) demonstrated the functions of OmpB on these steps. OmpA and OmpB contain species-specific epitopes that provide the basis for rickettsial serotyping by use of comparative indirect microimmunofluorescence assays (Parola et al., 2005). Genetic variation of the *ompA* gene allows the identification of various *Rickettsia* species (Roux et al., 1996). Gilmore et al. (1991) described heterogeneity in sequence of *ompB* gene of virulent and avirulent strains of *R. rickettsii*. Sequence analyses of the genes encoding OmpA and OmpB have been used as a tool for description of new rickettsial species (Niebylski et al., 1997; Bouyer st al., 2001; Jiang et al., 2005) and phylogenetic analyses (Fournier et at., 1998; Roux and Raoult, 2000; Moron et al., 2001; Stenos and Walker, 2000).

Rickettsia rickettsii ecology

Rickettsia rickettsii has as main reservoirs in nature hard ticks (family Ixodidae) of various genera and species, with which the agent has an intimate relationship, characterized by transovarial and transstadial transmission (McDade and Newhouse, 1986). Although *R. rickettsii* can also infect domestic and wild mammals, persistent maintenance of the agent does not occur, and the infection lasts for only a few days or weeks (Burgdorfer, 1988 cited by Labruna, 2009). Thus, vertebrate hosts cannot be considered reservoirs of *R. rickettsii* in nature. However, the participation of vertebrate hosts as amplifier of the rate of infection in the tick population by starting a new lineage

of infected ticks during rickettsemia is believed to be necessary, since the *R. rickettsii* pathogenicity for ticks precludes its enzootic maintenance solely by transovarial and transstadial transmissions in ticks (Labruna et al., 2009).

In ticks, *R. rickettsii* initially infects the epithelial cells of midgut, multiplies there, enters into the hemocoel, and invades and multiplies in other tick tissues including the salivary glands and ovaries. *R. rickettsii* can be found in tick hemocytes 3 to 5 days after a tick has fed on a rickettsemic animal, and all tick tissues can become infected with *R. rickettsii* as soon as 7-10 days after infectious feeding (Burgdorfer, 1977).

Species of ticks involved in transmission of *R. rickettsii* differ according to different geographic areas.

In the United States *Dermacentor andersoni* is the principal vector of *R. rickettsii* in the western states, as well as in Canada (Burgdorfer, 1969; McKiel, 1960), and *Dermacentor variablilis* is the principal vector in the eastern states (Sonenshine, 1979; Dumler and Walker, 2005). In Texas (USA), *A. americanum*, *Ixodes scapularis* and *Rhipicephalus sanguineus* were suspected to be involved in outbreaks of RMSF (Elliott et al., 1990). Recently, Demma et al. (2005) reported cases of RMSF in eastern Arizona, with common brown dog ticks, *Rhipicephalus sanguineus,* implicated as a vector.

In Mexico, *R. sanguineus* is the most important vector in western and central regions, and *A. cajennense* has been implicated in the southeastern region (Bustamante and Varela, 1947a).

Amblyomma cajennense is the most important vector of *R. rickettsii* in South America. It has been reported to be naturally infected in Panama (de Rodaniche, 1953), Colombia (Patino-Camargo, 1941), and Brazil (Dias e Martins 1939). Recently in Brazil, *Rhipicephalus sanguineus* was also reported as a suspected vector of *R. rickettsii* in an endemic area for Brazilian spotted fever in the metropolitan area of Sao Paulo, Brazil (Moraes-Filho et al., 2009), where *A. aureolatum* is a recognized vector (Pinter and Labruna, 2006).

In Costa Rica, *Haemaphysalis leporispalustris* has been demonstrated to participate in *R. rickettsii* ecology, since it has been isolated from this tick in endemic areas of RMSF (Fuentes et al., 1986; Hun et al., 2008). But a vector for *R. rickettsii* is not known in this country because *H. leporispalustris* is not a human-biting tick.

Amblyomma imitator

Amblyomma imitator Kohls was described by Kohls (1958) during re-examination of tick collections in the Rocky Mountain Laboratory that were labeled "*A. cajennense*", revealing a new species of tick, strongly resembling *A. cajennense* occurring in southern Texas (USA) and Mexico. Usually, the two species are distinguished by the presence of chitinous tubercles on the festoons of *A. cajennense* and the presence of projections over both sides of the apron of the genital aperture in *A. imitator* and by size, ornamentation, and the elongate ventral scutes of the *A. imitator* male. Hilburn et al. (1989) proposed the use of isozyme phenotypes for identification of these ticks. In this study the authors demonstrated eight enzymes that are diagnostic for the two species.

The distribution of *A. imitator* extends from southern Texas, southward through Mexico into Central America (Keirans and Durdenj, 1998). In Mexico, it is widely sympatric with *A. cajennense,* often found on the same host animal (Hilburn et al., 1989).

Hosts of *A. imitator* are various species of birds and mammals, including wild turkey, squirrels, peccary, dog, goat, horse, cattle, and humans (Keirans and Durden, 1998).

Interestingly, in 1933, Parker and colleagues performed tests of transmission of RMSF with supposed *A. cajennense*. In this study, stage to stage persistence of the agent was demonstrated from larvae through nymphs and into the resulting adults, as well as and transmission to guinea-pigs, showing successful transstadial transmission and that this tick was an efficient vector. However, the study of Kohls (1958) revealed that the ticks used for the mentioned tests were actually *A. imitator*, suggesting that this species of tick is a vector for *R. rickettsii.*

Recently, Medina-Sanchez et al. (2005) detected and isolated *R. prowazekii* from *A. imitator* ticks collected in Mexico. However, reports of *R. rickettsii* infecting this species have not been described in the literature.

Rocky Mountain spotted fever in Mexico

Rocky Mountain spotted fever (RMSF) is a life-threatening disease caused by *R. rickettsii* and is one of the most virulent infections identified in human beings.

The geographic distribution of RMSF is restricted to countries of the western hemisphere. The disease has been found in the USA, western Canada (McKiel, 1960) western and central Mexico (Bustamante and Varella, 1943; Bustamante and Varella, 1947b), Panama (Rodaniche and Rodaniche, 1950), Costa Rica (Fuentes, 1979), northwestern Argentina (Ripoll et al., 1999), Brazil (Piza, 1932; Dias e Martins, 1939), and Colombia (Patino et al., 1937).

Investigations of rickettsial diseases in Mexico including studies to identify vectors of *R. rickettsii* have been scarce. Cases of RMSF in Mexico occurred during the decades from 1930 to 1950. They were identified in Sinaloa, Sonora, Coahuila, and Durango states, where *Rhipicephalus sanguineus* ticks were incriminated as the vector (Bustamante, 1943; Mariotte et al., 1944; Bustamante and Varella, 1947a). An isolate of SFG *Rickettsia* was also established from *A. cajennense* ticks in the tropical Gulf Coast state of Veracruz (Bustamante and Varela, 1946). This isolate was demonstrated to cause periorchitis and scrotal necrosis in guinea pigs consistent with *R. rickettsii*.

In study carried out in 1996, sera of patients from Yucatan and Jalisco States, suspected clinically to have dengue fever were shown to contain antibodies reactive with *R. rickettsii* antigens by indirect immunofluorescence (Zavala-Velazquez et al., 1996). Since this year, epidemiologic surveillance was implemented to search for human cases in the public hospitals of Yucatan State, and in 2006 Zavala-Castro et al. reported the first human case of infection by *R. rickettsii* in this state, diagnosed by using immunohistochemistry and specific polymerase chain reaction. Subsequently, eight new cases of RMSF were reported in 2008 (Zavala-Castro et al., 2008).

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ARTICLE SUMMARY LINE:

In this study we report the natural infection of *Amblyomma imitator* ticks by *Rickettsia rickettsii* as well as its transovarial transmission by this species of ticks, strongly suggesting the discovery of a novel vector of *Rickettsia rickettsii* in South Texas and Mexico.

RUNNING TITLE:

Amblyomma imitator as a vector of *R. rickettsii*

Key Words: Ticks, *Rickettsia rickettsii*, RMSF, ultrastructure.

TITLE:

Amblyomma imitator: discovery of a novel vector for *Rickettsia rickettsii* in Mexico and South Texas

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ABSTRACT

Amblyomma imitator ticks collected from the field in Nuevo Leon State, Mexico, were maintained in the laboratory by feeding on naïve pathogen-free rabbits. Real-time PCR analysis of eggs laid from the first generation of laboratory-reared females indicated the presence of *Rickettsia* in some of the egg masses. Further characterization of the organism isolated from these ticks in Vero cells by analyzing fragments of *htrA, ompA* and *ompB* genes revealed it to be *R. rickettsii.* Ultrastructural analysis of tissues of adult ticks showed the presence of the rickettsial agent in midgut. This is the first report of the presence and transovarial transmission of *R. rickettsii* by naturally infected *A. imitator* ticks and suggests a role for this species of tick in transmission of Rocky Mountain spotted fever in Mexico and South Texas (USA).

INTRODUCTION

Rickettsioses, diseases of worldwide importance, are caused by small, Gram-negative, obligately intracellular bacteria that are transmitted to humans via ticks, fleas, mites or lice. The genus *Rickettsia* has been classically divided into two groups, the typhus group (TG) and the spotted fever group (SFG). In the United States and Mexico, transmission of Rocky Mountain spotted fever (RMSF) has been attributed to ticks of the genera *Dermacentor* (*D. variabilis* and *D. andersoni*), *Rhipicephalus* (*R. sanguineus*), and *Amblyomma* (*A. cajennense*).

Amblyomma imitator has close affinity with *Amblyomma cajennense* and was formerly confused with this species. Its distributional range extends from southern Texas (where it is more or less sympatric with *Amblyomma cajennense*), southward through Mexico (where it is widely sympatric with *A. cajennense*) into Central America (*1*). According to Kohls (*2*) it is apparent that the Rocky Mountain spotted fever transmission studies performed with supposed *A. cajennense* by Parker, Philip, and Jellison (*3*) were actually performed with *A. imitator*, which suggested a role for this species of tick as a vector of *R. rickettsii*. In the present study, we report the isolation and characterization of *R. rickettsii* from *A. imitator* using molecular biology methods, suggesting the discovery of a novel vector for the RMSF agent in Mexico and South Texas.

METHODS

Tick collection and establishment of colony: Ticks were collected from Nuevo Leon State Mexico (2007) and Laguna Atascosa National Wildlife Reserve in southern Texas (2009) using dry ice traps. The ticks were identified using morphological keys as defined by Kohls (2) and Keirans et al. *(1)*. The adult ticks from both localities were initially screened for the presence of Rickettsia using Gimenez staining of hemolymph content. The Nuevo Leon and South Texas collections were maintained in the laboratory at the UTMB, according to methods described by Brossard and Wikel (*4*). The colonies are maintained at 22^{o}C, under a 14 hour-light / 10 hour-dark photoperiod. Ticks are held in 16 ml glass vials (Wheaton Glass, Millville, NJ) with a mesh top over a super-saturated solution of potassium nitrate. The larvae and nymphs from the Nuevo Leon collection obtained blood meals from mice and adults were fed on naïve pathogen free rabbits.

DNA extraction and PCR: DNA from eggs of *A. imitator* ticks collected in Nuevo Leon state, Mexico and from 20 pools of 10 adult ticks from South Texas was extracted using a DNeasy Kit (Qiagen) following the recommendations of the manufacturer. For real-time PCR analysis, *Rickettsia*-specific primers CS5A and CS6 (*5*) that amplify a fragment of 150 bp of the *gltA* gene were utilized. The real-time PCR was performed in a Bio-Rad iCycler thermocycler with 25 µL per reaction, which contained 12.5 µL of the PCR mix, 0.4 mM of each primer and 10.5 µL of molecular-grade water/bovine serum albumin solution (BSA; 800 ng/µL) and 1 µL of each sample. *Rickettsia australis* DNA and water served as the positive and negative controls, respectively, and serial dilutions of a plasmid that contained the *R. prowazekii gltA* gene were utilized as the standards. Real-time PCR cycling conditions were as follows: 1 cycle at 95°C for 2 min, followed by 50 cycles of 15 s at 95°C, 30 s at 55°C, and 30s at 60°C. DNA from egg masses determined by real-time PCR to contain rickettsial DNA was used to select egg masses for isolation of the rickettsial agent. DNA extracted from ticks of South Texas collection was also used as template for a Nested-PCR using primers (*5*, *6*) for amplification of a fragment of the *htrA* gene using the following conditions: 94 ^{o}C for 15 min, 30 cycles at 95 ^{o}C for 1 min,

50 ^{o}C for 1 min, and 72 ^{o}C for 2 min, followed by a step at 72°C for 7 min.

Isolation of Rickettsia: Rickettsiae were isolated from an *A. imitator* egg mass in Vero cells using shell vials as previously described (*7*). In brief, egg masses from the first generation of laboratory-reared female ticks were collected and disinfected in 3% bleach and 70% ethanol, with washes with sterile PBS between each disinfection, and triturated in 200 µL of DMEM with 10% bovine calf serum (BCS) and utilized to inoculate Vero cell monolayers. The shell vials were incubated at 34°C and monitored daily by Diff-Quik staining for the presence of rickettsiae. Slides that contained four rickettsiae were considered positive, and the monolayer from the corresponding shell vial was removed manually and used to inoculate a T-25 flask containing Vero cells (in DMEM containing 3% BCS) for propagation of the agent. Cells of the 25-cm^2 flask were observed by Diff-Quik staining until more than 90% of the cells were infected, when they were harvested and inoculated into 150-cm^2 flasks of Vero cells. The *A. imitator* isolates were genotypically identified by sequencing the PCR product of the resultant infected cells, as described below.

IFA: Indirect immunofluorescence assay was performed from infected-Vero cells using rabbit polyclonal antibody against *R. rickettsii* Sheila Smith strain (diluted 1:200) and fluorescein isothiocyanate-labeled goat anti-rabbit IgG (diluted 1:300) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) following the Protocol for Identification of Rickettsial Antigens of the Rickettsial and Ehrlichial Diseases Research Laboratory at University of Texas Medical Branch (UTMB) with some modifications. *Rickettsia rickettsii*-infected Vero cells were used as the positive control, and uninfected Vero cells were the negative control. Slides containing a smear from infected and uninfected Vero cells were fixed in acetone for 10 minutes, rinsed with PBS, incubated with primary antibody diluted in PBS/BSA at 34°C in a humid chamber for 30 minutes, washed twice with PBS solution containing Evans Blue and Triton X-100 (0.1%), and incubated with the secondary antibody diluted in PBS at 34°C in a humid chamber for 30 minutes. Slides were mounted with Crystal Mount (Biomedia, Foster City, CA) under coverslips and examined using a fluorescent microscope Olympus BX31.

Characterization of the rickettsial isolate: For detection and characterization of the organism isolated in Vero cells, partial sequences of the *Rickettsia*- specific genes *htrA,* o*mpB*, and o*mpA* were amplified and analyzed. DNA from infected Vero cells was

extracted using the DNeasy Kit (Qiagen). Nested-PCR was performed using *Rickettsia*-specific primers 17K3 and 17K5 (*5*) for the first reaction and 17KD1 and 17kD2 described by Webb et al. (*6*) for the second reaction for amplification of fragments of 547 bp and 434 bp, respectively, of the *htrA* gene. For amplification of a fragment of 856 bp of the *ompB* gene the pair of primers 120-M59 and 120-807 was used *(8)*. A 533 bp fragment of the *ompA* gene was amplified using the primers Rr190.70F and Rr190.602R (*8*). For one of the samples a semi-nested PCR was necessary to amplify an *ompA* fragment. The primers Rr190.70F and Rr190.701R (*10*) were used for the first reaction, and primers Rr190.70F and Rr190.602R for the second reaction. The sequences of primers used for characterization are shown in the table.

For sequencing, amplified fragments were cloned into pCR®4-TOPO® (Invitrogen TOPO TA Cloning® Kit) following the manufacturer's protocol. Plasmid DNA extraction was performed using Wizard Plus SV Minipreps DNA Purification System (Promega). Plasmids containing inserts were sequenced at least three times using the Universal primers M13F and M13R. The nucleotide sequences were edited with SeqMan and compared with the corresponding homologous sequences available at GenBank, using Blast analysis *(11)*.

Electron Microscopy: For detection of rickettsiae in *A. imitator* salivary glands, midguts and ovaries were dissected from unfed adults and fixed in modified Ito's fixative (2.5% formaldehyde, 0.1% glutaraldehyde, 0.03% trinitrophenol, 0.03% $CaCL_2$ in 0.05M cacodylate buffer at pH 7.3-7.4) (*12*), postfixed in 1% osmium tetroxide for 1 h, stained *en bloc* in 2% aqueous uranyl acetate for 20 minutes at 60°C, dehydrated in ethanol, and embedded in epoxy resin (Poly/Bed 812). Ultrathin sections were cut on a Reichert Ultracut S ultramicrotome, placed on copper grids, stained with lead citrate, and examined in a Philips (FEI) CM-100 electron microscope at 60kV.

RESULTS

Detection and Characterization of Rickettsia

In 2007,we collected five tick species (*A. imitator*, *Boophilis microplus*, *Dermacentor variabilis*, *Rhipacephalus sanguineu*s, and *Ixodes* species) from the field in Nuevo Leon State (24°50'N, 100°04'W), Mexico.(*13*) From this collection we were

only able to successfully establish a colony of *A. imitator* that was maintained in the laboratory by allowing to blood feed on naïve rabbits. Real-time PCR analysis of eggs laid by one full generation of laboratory-reared females indicated the presence of rickettsial DNA in two egg masses (Figure 1). The CT value for these samples was high (38 cycles), indicating a low concentration of *Rickettsia* in the egg masses. The organisms were isolated in Vero cells from two egg masses that showed the presence of rickettsial DNA by real-time PCR. Rickettsial organisms were observed in Vero cells by using Diff-Quik staining and IFA with antibody against *R. rickettsii* (Figs. 2 and 3). Rickettsiae were also detected in Vero cells and characterized by analyzing the sequences of fragments of 434 bp, 856 bp and 533 bp amplified from *Rickettsia*-specific genes, *htrA, ompB*, and *ompA,* respectively. Amplification and analysis of the *ompB* fragment were achieved from only one of the samples.

Analysis of the sequences obtained from the *htrA* gene fragments amplified from both samples showed 99% identity with spotted fever group *Rickettsia* sequences, including the *Rickettsia rickettsii* sequence from a fatal case of RMSF in southwestern Mexico, Yucatan State (DQ176856.1) (*14*), with which the alignment of the sequences obtained from the samples of the present study presented the highest Bit Score (S), with only one nucleotide difference. Analysis of the partial sequence of *ompB* gene showed 100% identity between the organism isolated from one of the egg masses and *R. rickettsii* Sheila Smith strain (CP000848.1). The partial sequences obtained from the *ompA* gene of both isolates were 100% identical to the corresponding sequence of *R. rickettsii* Sheila Smith strain (CP000848.1) in GenBank. The sequences obtained in this study were submitted to the GenBank with the following accession numbers: (GU723476) and (GU723477) for the fragments amplified from *htrA* gene, (GU723478) and (GU723479) for the fragments of *ompA* gene and (GU723475) for the fragment of *ompB* gene.

In addition, we have obtained preliminary data from *A. imitator* ticks collected from the field in Laguna Atascosa National Wildlife Reserve in southern Texas in 2009. The sequence analysis of a 434 bp fragment of the *htrA* gene amplified from DNA extracted from two pools of ticks using the primers listed previously showed 100% and

99% identity with *R. rickettsii* strain from Mexico (DQ176856.1). Isolation and further characterization of the agent(s) infecting ticks collected in this area are in progress.

Ultrastructural Observations

Ultrathin sections of salivary glands, ovaries and midguts of unfed PCR-positive *A. imitator* were examined in a transmission electron microscope. Those ticks were tested for the presence of *Rickettsia* by nested-PCR with primers that amplify a 434 bp fragment of the *htrA* gene and were shown to contain DNA of *Rickettsia*. Single rickettsial cells were found in highly vacuolated cytoplasm of midgut epithelial cells of one tick (#5 – male) (Fig. 4A). They had typical ultrastructure for Gram-negative bacteria being surrounded by two trilaminar membranes (Fib. 4B): inner cytoplasmic membrane and outer cell wall membrane. Their size was 0.6 x 0.2 μm.

DISCUSSION

This study demonstrated that *R. rickettsii* has been isolated from egg masses of *A.imitator* ticks that were documented to contain *R. rickettsii* DNA by real-time PCR and DNA sequencing. Since the eggs were laid by field collected unfed adult ticks that were further fed in the laboratory using naïve pathogen-free rabbits for one full generation in order to establish a colony of ticks, the presence of those organisms in eggs documents their transovarial transmission by naturally infected ticks, suggesting a role for this species of ticks in the maintenance of *R. rickettsii* in nature.

Since the hosts of *A. imitator* are various species of birds and mammals, including wild turkey, squirrel, peccary, dog, goat, horse, cattle, and humans (*1*), and the adult stage of this tick species seems to be very aggressive to human beings (*13*), the medical importance of the findings of this study is the potential participation of this tick in a zoonotic cycle of *R. rickettsii*. Because of its high similarity to *A. cajennense* and the overlapping range of distribution of these species of ticks in Mexico, it is likely that the previously described spotted fever group rickettsial isolate from supposed *A. cajennense* in Veracruz State, which was demonstrated to cause periorchitis and scrotal necrosis in guinea pigs consistent with *R. rickettsii* (*15*), was actually isolated from *A. imitator*. Kohls (*2*), when re-examining tick collections in the Rocky Mountain

Laboratory that were labeled "*A cajennense*", identified *A. imitator* and suggested that the positive results in tests of transmission of RMSF performed in 1933 (*3*) using supposed *A. cajennense* were actually obtained using *A. imitator*. Evaluation of transmission of *R. rickettsii* by this species of tick is in progress in our laboratory.

Cases of RMSF in Mexico occurred during the decades from 1930 to 1950, and *Rhiphicephalus sanguineus* ticks were incriminated as the vector (*16*, *17*, *18*). In a study carried out in 1996, sera of patients from Yucatan and Jalisco States suspected clinically to have dengue fever were shown to contain antibodies reactive with *R. rickettsii* antigens by indirect immunofluorescence (*19*). Subsequently, the first human case of infection with *R. rickettsii* in Yucatan State was reported in 2006, diagnosed by using immunohistochemistry and specific polymerase chain reaction (*14*). With 99% identity and only one nucleotide difference in the sequence of a fragment of the *htrA* gene obtained in this study (*R. rickettsii* strain from Mexico, DQ176856.1) and the corresponding sequence obtained in the present study showing the closest relatedness, the close relationship between these strains is apparent. It would be interesting to have the availability of the sequences of *ompB* and *ompA* genes of the Yucatan strain for comparison. Although the sequences of the fragments of these genes obtained here showed 100% identity to *R. rickettsii* Sheila Smith strain, it would not be unlikely to have the same result in comparison of them and the *R. rickettsii* strain from Mexico, (DQ176856.1), since this strain also has a close relationship with *R. rickettsii* Sheila Smith strain. Studies determining the species of tick with potential for transmission of *R. rickettsii* are necessary in Mexico, and according to the results of the present study, we suggest that *A. imitator* is a potential vector or at least is involved in maintenance of this rickettsial agent in nature in this country as well as in South Texas (US).

The ultrastructure of *Rickettsia rickettsii* observed in this study, which includes the absence of a discrete microcapsular layer and electron lucent halo zone, are similar to those in the study of Hayes and Burgdorfer *(20).* Through electron microscopic studies of *R. rickettsii*, the authors observed that physiological conditions, under which ticks actively feeding and obtaining essential nutrients, metabolites and temperature required for rickettsial growth, influence the ultrastructural appearance of the

microcapsular and electron lucent zone of *R. rickettsii*. Their study correlated the ultrastructural features of the rickettsia and electron lucent zone to the phenomenon of reactivation of virulence of this bacterium. The growth-limiting conditions in such reactivation are well described by Spencer and Parker (*21*). As emphasized by Hayes and Burgdorfer (*20*), "the ability of *R. rickettsii* to reestablish its surface structures (microcapsular layer and electron lucent zone) under optimal physiological conditions after having lost them during the periods of stress and starvation of its arthropod host may indeed provide the basis for reactivation and restoration of virulence".

Transmission electron microscopy of the midgut ultrathin sections revealed rickettsia with typical ultrastructure though in low quantities and only in one tick.

CONCLUSION

Transmission of RMSF has been attributed to ticks of the genera *Dermacentor* (*D. variabilis* and *D. andersoni*), *Rhipicephalus* (*R. sanguineus*), and *Amblyomma* (*A. cajennense*) in the United States and Mexico. In this study we report the natural infection of *A. imitator* by *R. rickettsii* as well as transovarial transmission by this species of ticks, strongly suggesting the discovery of a novel vector of *R. rickettsii* in Mexico and South Texas.

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BIOGRAPHICAL SKETCH

K.A. Oliveira has a degree in Nutrition from the Federal University of Vicosa and a Master's degree in the Program of Agricultural Biochemistry of the same University with emphasis in molecular biology and research in infectious diseases transmitted by

ticks. Currently, she is pursuing a PhD degree at the same University investigating rickettsioses.

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Table. Primers used for amplification of rickettsial genes

Genes and primers	Primer sequence (5' →3')	References
gltA		
CS5A	GAGAGAAAATTATATATCCAAATGTTGAT	(*4*)
CS6	AGGGTCTTCGTGCATTTCTT	(*4*)
htrA		
17K3	TGTCTATCAATTCACAACTTGCC	(*4*)
17K5	GCTTTACAAAATTCTAAAAACCATATA	(*4*)
17KD1	GCTCTTGCAACTTCTATGTT	(*6*)
17KD2	CATTGTTCGTCAGGTTGGCG	(*6*)
ompB		
120-M59	CCGCAGGGTTGGTAACTGC	(*7*)
120-807	CCTTTTAGATTACCGCCTAA	(*7*)
ompA		
Rr190.70F	ATGGCGAATATTTCTCCAAAA	(*8*)
Rr190.602R	AGTGCAGCATTCGCTCCCCCT	(*8*)
Rr190.701R	GTTCCGTTAATGGCAGCATCT	(*9*)

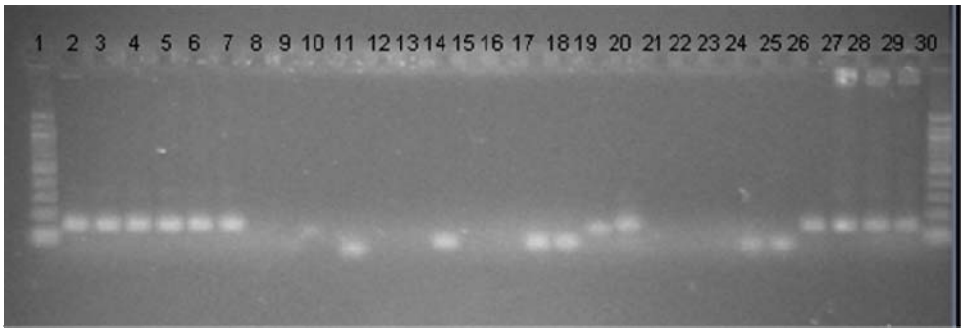

Figure 1. Agarose gel (1,2%) showing citrate synthase gene fragments amplified by real-time PCR from DNA of egg masses. Lanes 1 and 30: 100bp DNA ladder; lanes 2-7: standards (serial dilutions of plasmids containing *Rickettsia prowazekii gltA* gene); lane 8: negative control; lanes 9 to 26: products amplified from egg masses; lanes 27 to 29: positive control.

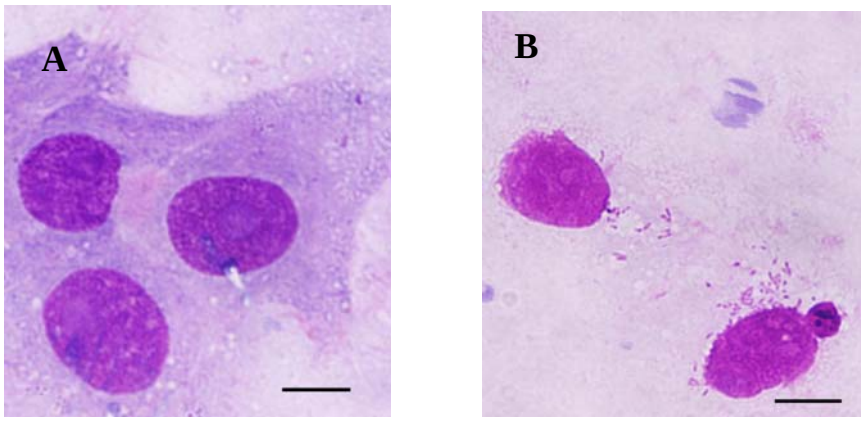

Figure 2: Dif-Quik stain showing *Rickettsia rickettsii* in cultured Vero cells. A. Non-infected Vero cells. B. Infected Vero cells. Bars: 10 µm.

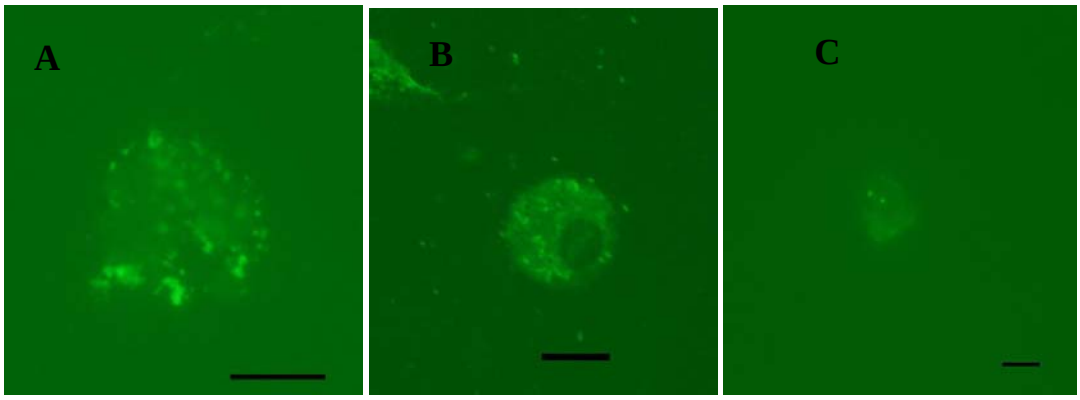

Figure 3: Indirect immunofluorescence of cultured Vero cells infected and uninfected with *R. rickettsii* using rabbit polyclonal Ab against *Rickettsia rickettsii* Sheila Smith strain (1:200) and fluorescein isothiocyanate-labeled goat anti-rabbit IgG. A. Rickettsial organisms isolated from a sample of *A. imitator* egg masses in this study infecting the cytoplasm of Vero cell. Bar: 10 µm. B and C. Positive and negative controls: Vero cells infected and non-infected with *Rickettsia rickettsii*, respectively. Bars: 20 µm.

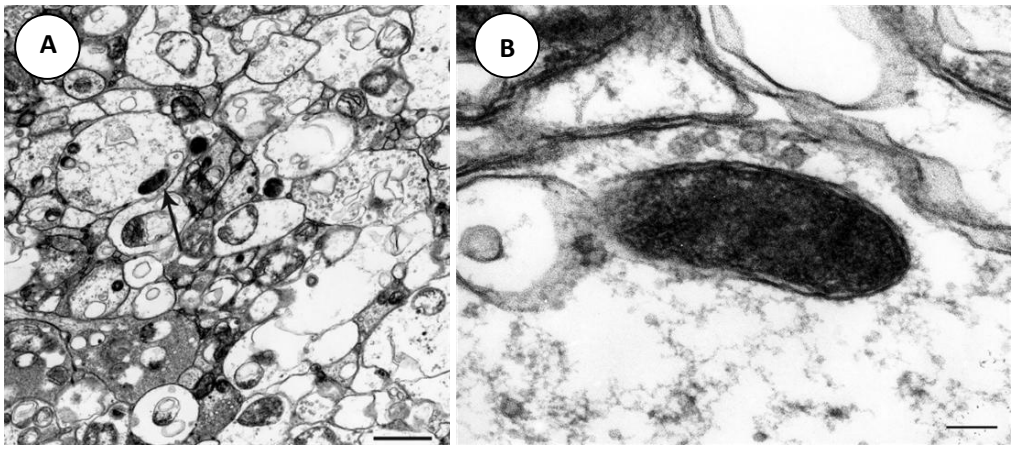

Figure 4: Electron microscopy of tick tissue showing *Rickettsia rickettsii* in a cell of tick midgut. A. Bar: 1 µm; B. The trilaminar cell wall is separated from the cell membrane by the periplasmic space. Bar: 0.1 µm.