ORIGINAL ARTICLE

Isolation of *Rickettsia amblyommatis* in HUVEC line

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Abstract

Rickettsia amblyommatis, formerly named *Rickettsia amblyommii* and '*Candidatus* Rickettsia amblyommii' is an intracellular bacterium belonging to the spotted fever group *Rickettsia*. It is highly prevalent in *Amblyomma americanum* and in other *Amblyomma* spp. throughout the Western Hemisphere. *R. amblyommatis* has been cultivated in chicken fibroblast, primary embryonated chicken eggs, Vero cells and arthropod-derived cells. Because of the affinity of rickettsiae to invade vascular endothelial cells, we tried to isolate *R. amblyommatis* from a nymph of *Amblyomma cajennense* s.l. collected in Saltillo (Coahulia, Mexico) using human umbilical vein endothelial cells (HUVEC). One tick half was analysed by *ompA* PCR and was found to be positive for *R. amblyommatis*. The other half was selected for *in vitro* culture of *Rickettsia* spp. It was triturated in 1 mL of endothelial cell growth medium with 1% antibiotic–antimycotic solution, and the homogenate was inoculated into a HUVEC line. Culture was maintained at 33°C in endothelial cell growth medium plus 2 mM L-glutamine and 2% fetal calf serum, with 5% CO_2. The medium was changed weekly. Culture was checked by Gimenez stain for *Rickettsia*-like intracellular organisms. After 48 days of incubation, *Rickettsia*-like organisms were observed in HUVEC. PCR assays and sequencing of *ompA* gene in the culture suspension showed 100% identity with *R. amblyommatis*. This isolate was successfully established in HUVEC, and it has been deposited in the collection of the Center of Rickettsioses and Arthropod-Borne Diseases, Infectious Diseases Department, Hospital San Pedro–Center of Biomedical Research from La Rioja, Logroño, Spain. The HUVEC line is a useful tool for the isolation of *R. amblyommatis*.

Keywords: *Amblyomma cajennense*, *Candidatus* Rickettsia amblyommii, HUVEC line, *Rickettsia amblyommatis*
Original Submission: 15 November 2017; **Accepted:** 5 December 2017
Article published online: 9 December 2017

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Introduction

Rickettsia amblyommatis is an intracellular bacterium belonging to the spotted fever group *Rickettsia*. It was isolated from an *Amblyomma americanum* adult tick collected from vegetation in the US state of Tennessee in 1973 and was designated as strain WB-8-2 ^{T} [1,2]. In 1995, Stothard [3] characterized that strain and a new one also detected in *A. americanum* (strain MO 85-1084) by molecular tools. The *rrs* sequence was similar to others in the spotted fever group *Rickettsia*. Nevertheless, analysis of the 17 kDa gene indicated that WB-8-2 ^{T} and MO 85-1084 were different from other known species of the genus [3]. From 1995 to 2016, it was named as *Rickettsia amblyommii* and '*Candidatus* Rickettsia amblyommii' in the scientific literature, although these names have never been validated. In 2016, Karpathy et al. proposed the novel species name *R. amblyommatis,* which confirmed to the rules of the International Code of Nomenclature of Prokaryotes [4]. This bacterium is highly prevalent in *A. americanum,* and it has been also detected in other *Amblyomma* species throughout the Western Hemisphere as *Amblyomma maculatum* in the United States [5], and *Amblyomma auricularium, Amblyomma cajennense, Amblyomma coelebs, Amblyomma geayi, Amblyomma humerale, Amblyomma longirostre, Amblyomma mixtum, Amblyomma neummanni, Amblyomma hadanii, Amblyomma oblongoguttatum, Amblyomma ovale, Amblyomma sculptum* and *Amblyomma tonellidae* in Central and South

https://doi.org/10.1016/j.nmni.2017.12.002

America [6–19]. Nowadays, several of these *Amblyomma* species are within *A. cajennense* s.l., because this taxon has been recently reassessed, including *A. cajennense* sensu stricto, *A. mixtum, A. sculptum, Amblyomma interandinum, A. tonellidae* and *Amblyomma patinoi* [20].

R. amblyommatis has never been confirmed as a human pathogen, although some serologic evidence suggests that humans develop an immune response to this organism and it may be associated with disease manifestations in some patients [21,22]. It has been demonstrated that an isolate from a Costa Rican strain of *'Ca.* R. amblyommii' causes fever and pathologic signs of disease in guinea pigs [23].

To date, *R. amblyommatis* has been cultivated in chicken fibroblast, primary embryonated chicken eggs, Vero cells, the mosquito cell Sua5B and the tick cells ISE6 and AAE2 [6,24,25].

In an attempt to prove the usefulness of human umbilical vein endothelial cells (HUVEC) for the isolation and maintenance of *Rickettsia* spp., we tried to isolate *R. amblyommatis* from a nymph of *A. cajennense* s.l. collected in Saltillo (Coahulia, Mexico).

Materials and methods

A nymph of *A. cajennense* s.l. collected in Saltillo in June 2014 was sent to the Center of Rickettsiosis and Arthropod-Borne Diseases (Infectious Diseases Department, Hospital San Pedro–Center of Biomedical Research from La Rioja, Logroño, Spain). The tick was genetically identified using PCR assays targeting the mitochondrial 12S rRNA and 16S rRNA fragment genes [26,27]. The obtained sequences showed the highest identities (99.7% and 99%, respectively) with *A. cajennense* s.l. sequences from GenBank (accession no. JX987841 and KX544819). Moreover, the 16S rRNA nucleotide sequence also showed the same identity with the sequence from a tick specimen classified as *A. mixtum* (GenBank accession no. KT820359). The arthropod was surface sterilized by immersion in 1% benzalkonium chloride for 5 minutes and 70% ethanol for 1 minute, and rinsed twice with sterile distilled water [28]. One tick half analyzed by *ompA* PCR was found to be positive for *R. amblyommatis* [29,30]. The other half was selected for *in vitro* culture of *Rickettsia* spp. It was triturated in 1 mL of endothelial cell growth medium (Sigma-Aldrich) with 1% antibiotic–antimycotic solution (Gibco), and the homogenate was inoculated into a HUVEC line. Culture was maintained at 33°C in endothelial cell growth medium plus 2 mM L-glutamine and 2% fetal calf serum, with 5% CO_2 atmosphere. For the first 3 days, 100 U/mL penicillin and 100 µg/mL streptomycin were also added. The medium was changed weekly, and culture by Gimenez stained to check for *Rickettsia*-like intracellular organisms. When the staining method was positive, *ompA* PCR and sequencing was used to confirm the *Rickettsia* species in the cells. Two negative controls, one that used water instead of template DNA and the other that used template DNA but no primers, as well as a positive control of *Rickettsia slovaca* strain S14ab DNA (from the collection of the Center of Rickettsiosis and Arthropod-Borne Diseases), were included in all PCR assays. Passages onto fresh, uninfected cells were performed, and aliquots of infected subcultures were also tested by PCR.

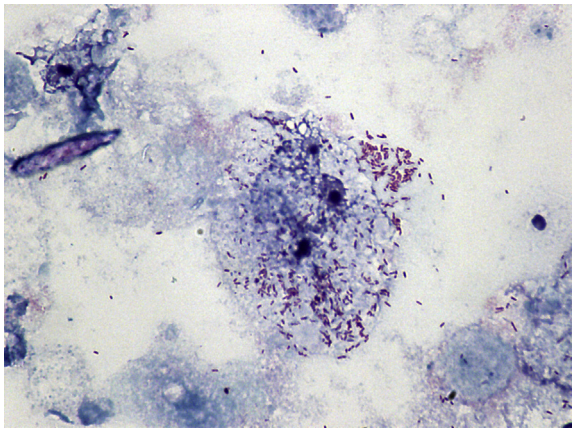

FIG. 1. Gimenez-stained cytocentrifuge smear (100X magnification) showing infection of human umbilical vein endothelial cell with *Rickettsia amblyommatis* at day 48 after inoculation with *Amblyomma cajennense* tick homogenate.

Results

After 48 days of incubation, intracellular *Rickettsia*-like organisms were observed in HUVEC using Gimenez stain (Fig. 1). PCR assays and sequencing of the *ompA* gene in culture suspension showed 100% identity with *R. amblyommatis* (GenBank accession no. CP003334). The bacteria were taken through three subcultures in HUVEC, and the *ompA* sequence obtained by PCR carried out at passage 6 was identical to that of the original isolate. This isolate was successfully established in HUVEC, and it has been deposited in the collection of the Center of Rickettsiosis and Arthropod-Borne Diseases (*R. amblyommatis* strain 4Me).

Discussion

Our results correspond to the first isolation of *R. amblyommatis* from an infected *A. cajennense* s.l. tick in the HUVEC line.

HUVEC comprise the same ontogenetic type of cells which rickettsiae parasitize *in vivo*. Consequently, these cells are widely used as a model system for studying rickettsia–host cell

interactions *in vitro* [31–36], but they have been little used for isolating rickettsia species [37].

R. amblyommatis had been previously cultivated in chicken fibroblast, primary embryonated chicken eggs, Vero cells and the arthropod-derived lines ISE6, AAE2 and Sua5B [6,24,25]. The mosquito cell line Sua 5B has been used to isolate *R. amblyommatis* from wild specimens of *A. americanum*. Infection was stable in the cells for over 40 passages with no decrease in the cell infection rate, which shows this mosquito cell can be highly effective for isolating and cultivating *Rickettsia* from ticks [24], and it is known that tick cell lines are effective for the isolation of *Rickettsia* spp. [25,38–42]. Nevertheless, the isolation of *R. amblyommatis* in an endothelial cell line gives us a new tool for the isolation of rickettsia because this cell line has shown a high permissiveness to infection with this intracellular bacterium; it has also shown advantages over other cell lines using standard, commercially available media.

R. amblyommatis has never been directly detected in human clinical samples, although there has been serologic evidence in the United States that this rickettsial agent could cause spotted fever illness [21,22]. In addition, *R. amblyommatis* was detected in a tick that subsequently caused rash at the bite site in a patient without other symptoms [43].

The nymph of *A. cajennense* s.l. infected with *R. amblyommatis* was collected in Saltillo, a region located in the northern Mexico on the border with the US state of Texas. In Mexico, *R. amblyommatis* has been detected in *A. mixtum* (*A. cajennense* s.l.) detached from people [19].

R. amblyommatis may also play a role in the ecology and epidemiology of other pathogenic spotted fever group rickettsiae because *A. americanum* is a potential vector of at least two confirmed rickettsial pathogens, *Rickettsia rickettsii* and *Rickettsia parkeri,* and it is possible that the observed high rates of *R. amblyommatis* infection could inhibit the transovarial transmission of these pathogenic rickettsiae [44]. Rocky Mountain spotted fever (RMSF) is an emerging public health concern in the United States and near the US–Mexico border, a site that recently saw several fatal cases of RMSF. In all cases, infection was caused by *R. rickettsii* [45]. Nevertheless, there have been suspected cases of RMSF where the causative agent, *R. rickettsii,* was not identified in the local tick population. In these areas, patients with clinical signs of RMSF had low or no detectable antibodies to *R. rickettsii,* resulting in an inability to confirm a diagnosis. On the other hand, there are seroepidemiologic studies that indicate that humans are being exposed to *R. amblyommatis,* and this species might be responsible for cases classified as RMSF [21,46].

There are cases of RMSF that correspond to the geographic range of *A. americanum*. In these areas, it has been suggested that reports of RMSF are more likely due to other *Rickettsia* spp. [47,48].

Because *R. amblyommatis* is suspected to be a human pathogen, the availability of cell lines of proven effectiveness in the isolation of this microorganism allows us to characterize this bacterium. The development of culture systems for the growth of *Rickettsia* is critical to the genetic and antigenic evaluation of pathogenic and nonpathogenic species.

Acknowledgements

We are grateful to A. Díaz Castaño, Centro Hospitalario La Concepción, Saltillo, Coahuila, Mexico, for providing ticks. We would like to acknowledge the financial support of 'Fondo Europeo de Desarrollo Regional'.

Conflict of interest

None declared.

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