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Short communication

'*Candidatus* Rickettsia nicoyana': A novel *Rickettsia* species isolated from *Ornithodoros knoxjonesi* in Costa Rica

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ABSTRACT

Rickettsiae are intracellular bacteria commonly associated with hematophagous arthropods. Most of them have been described in hard ticks, but some have been found in soft ticks. Here we report the detection and isolation of a new *Rickettsia* from *Ornithodoros knoxjonesi* larvae collected from *Balantiopteryx plicata* (Emballonuridae) in Nicoya, Costa Rica. Two ticks were processed to detect *Rickettsia* spp. genes *gltA*, *ompA*, *ompB*, and *htrA* by PCR. Part of the macerate was also inoculated into Vero E6 and C6/36 cell lines, and cells were evaluated by Giménez stain, indirect immunofluorescence assay (IFA), and PCR. Both ticks were positive by PCR and rickettsial growth was successful in Vero E6 cells. Amplification and sequencing of near full length *rrs*, *gltA*, *sca4* genes, and fragments of *ompA* and *ompB* showed that the *Rickettsia* sp. was different from described species. The highest homologies were with '*Candidatus* Rickettsia wissemanii' and *Rickettsia peacockii:* 99.70% (1321/1325) with both sequences for *rrs*, 99.58% (1172/1177) and 99.76% (1246/1249) for *gltA*, 99.26% with both sequences (2948/2970 and 2957/2979) for *sca4*, 98.78% (485/491) and 98.39% (2069/2115) for *ompA*, and 98.58 (1453/1474) and 98.92% (1459/1475) for *ompB*; respectively. Bat blood, spleen, liver, and lung samples analyzed for *Rickettsia* detection were negative. Results demonstrate that the *Rickettsia* isolated from *O. knoxjonesi* is probably an undescribed species that belongs to the spotted fever group, for which '*Candidatus* Rickettsia nicoyana' is proposed. Considering that *B. plicata* inhabits areas where contact with humans may occur and that human parasitism by *Ornithodoros* has been reported in the country, it will be important to continue with the characterization of this species and its pathogenic potential.

1. Introduction

The genus *Rickettsia* is comprised of obligate Gram-negative intracellular bacteria of arthropods (e.g., ticks, mites, fleas, and lice), some of which are considered endosymbionts while others are known pathogens of humans and other vertebrates (Rounds et al., 2012; Parola et al., 2013). In this respect, several rickettsiae that were initially considered non-pathogenic have been implicated in human infections and disease, and new species of *Rickettsia* continue to be identified in which their pathogenicity is yet to be determined (Parola et al., 2013).

Although the majority of known *Rickettsia* species have been described from hard ticks (Ixodida: Ixodidae), several species have been found in association with soft ticks (Ixodida: Argasidae). For instance, *Rickettsia hoogstraalii* and similar genotypes have been detected in *Carios capensis*, and other species of *Carios*, *Argas*, and *Ornithodoros* in USA, Japan, Croatia, and several African countries (Cutler et al., 2006; Duh et al., 2010; Pader et al., 2012; Dietrich et al., 2014; Lafri et al., 2015). This species has also been detected in hard ticks (*Haemaphysalis* spp.) from Spain (Portillo et al., 2008). Additionally, *Rickettsia lusitaniae* was isolated from *Ornithodoros erraticus* from Portugal (Milhano et al., 2014), and '*Candidatus* Rickettsia wissemanii' was described from *Ornithodoros hasei* in French Guiana (Tahir et al., 2016). Other undescribed rickettsiae have also been found in *Argas* and *Carios* (Socolovschi et al., 2012; Loftis et al., 2005). The pathogenic potential of these recently described rickettsiae is unknown.

To date, there are no reports of rickettsiae associated with soft ticks from Central America. In Costa Rica, one study by Vargas in 1984 reports the occurrence of *Ornithodoros* associated with bats and refers to their importance since several people were bitten by

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these ticks. However no analysis for *Rickettsia* presence was made (Vargas, 1984). There are more recent reports concerning distribution and human or animal parasitism by soft ticks in Central America, although none of them included the detection of rickettsiae (Rangel and Bermudez-Castillero, 2013; Bermúdez et al., 2013; Bermúdez et al., 2015; Venzal et al., 2015). Therefore, the aim of this paper is to report the detection and isolation of a putative new species of *Rickettsia* from *Ornithodoros knoxjonesi* collected from *Balantiopteryx plicata* (Emballonuridae) bats in Costa Rica.

2. Materials and methods

2.1. Tick identification and detection of rickettsiae

Three soft tick larvae were collected in June 2014, from two *Balantiopteryx plicata* bats in Nicoya (10° 10′ 10.342" N, 85° 26′ 56,538" W), Guanacaste, Costa Rica. Bats were captured using mist nets surrounding their roosting habitat and collected following the recommendations of the Institutional Committee of Care and Use of Animals of the University of Costa Rica (CICUA-36-13), according to international animal welfare standards. For morphological evaluation of the larvae, one tick was cleared in lactophenol and mounted on a glass slide using Hoyer's medium according to established processing methods for mites (Krantz and Krantz, 2009); identification was made following keys for immature argasid ticks of the Neotropical Region (Barros-Battesti et al., 2013).

The two remaining ticks were processed individually to determine the presence of *Rickettsia* sp. First, the larvae were washed in 0.02% iodine and 70% ethanol solution for 10 min, then washed three times in sterile distilled water, macerated, and resuspended in 1 ml of brain-heart infusion broth (Hun et al., 2011). Genomic DNA was extracted from 200 μl of each macerate using Nucleospin Tissue® kit (Macherey-Nagel), following manufacturer's instructions. To detect *Rickettsia* spp. DNA, PCRs targeting four different genes were performed: *gltA* using primers CS-78 and CS-323 (401 bp product) (Labruna et al., 2004); *ompA* by a semi-nested PCR using primer pairs Rr190-70/Rr190-701 and Rr190-70/Rr190-602 (532 bp product) (Regnery et al., 1991; Roux et al., 1996); *ompB* using primers 120-M59f and 120-807 (856 bp product) (Roux and Raoult, 2000); and *htrA* by a nested PCR using primer pairs R17-122/R17-500 and TZ15/TZ16 (246 bp product) (Tzianabos et al., 1989; Blair et al., 2004). Blood, spleen, liver, and lungs from both bats were processed to detect the *Rickettsia gltA* and *ompB* gene fragments as stated previously. All the products were visualized on a 2% agarose gel after Gel Red staining, under UV light with a ChemiDocTM XRS+ System (BIO-RAD).

For molecular confirmation of tick species, extracted DNA was used to amplify the 16S mitochondrial rDNA gene using primers 16S + 1 and 16S-1 (460 bp product), according to methods described previously (Black and Piesman, 1994; Mangold et al., 1998).

PCR products of ticks and rickettsiae were purified and sent to Macrogen Inc. (Seoul, South Korea) for sequencing with the corresponding primers (the second pair of primers was used in nested or semi-nested PCRs). DNA sequences were edited and assembled with DNA Baser (Heracle BioSoft S.R.L.), and a sequence homology search was performed against the NCBI database using BLAST.

2.2. Isolation of Rickettsia sp. in cell culture

Isolation was attempted from the macerate of the two tick larvae by inoculating 200 μl of the macerate into confluent monolayers of C6/36 (ATCC CRL-1660) and Vero E6 (ATCC CRL-1586) cell lines, according to previously described methods (Hun et al., 2011). Cells were maintained in RPMI or Minimal Essential Medium (MEM), respectively, supplemented with 5% fetal bovine serum, and incubated at 28 °C in a 5% CO_2 atmosphere. The presence of *Rickettsia* spp. was monitored for 4 weeks by observing the appearance of a cytopathic effect and by Gimenez staining (Giménez, 1964). To confirm the identification of the isolates, DNA was extracted from positive cell cultures as stated above and PCR reactions were carried out to amplify the same four *Rickettsia* genes. The products were processed in the same manner and sequences were edited and compared to the ones obtained directly from the ticks.

Aliquots of the infected Vero E6 cells were treated according to methods previously described in order to prepare antigens on glass slides for testing by an indirect immunofluorescence assay (IFA) (Walker et al., 1977). Guinea pig sera reactive against *Rickettsia rickettsii* (end titer: 4 096) and *Rickettsia felis* (end titer: 512) were used at 2-fold serial dilutions to determine their end titers against the new *Rickettsia* isolate (Rivas et al., 2015). Anti-guinea pig IgG produced in goat and conjugated to fluorescein isothiocyanate (FITC) (Sigma–Aldrich) was used as secondary antibody at a dilution of 1:128. Negative antigen controls were included using Vero E6 mock infected cells.

2.3. Identification of Rickettsia sp.

To identify the species of *Rickettsia*, the *rrs*, *gltA,* and *sca4* genes were amplified from isolate DNA using primers previously described: 27F and 1492R for *rrs* (~1 465 bp sequence); CS2d, CSEndr, CS890r, and Rp877p for *gltA* (~1 270 bp sequence), and D1f, D928r, D767f, D1390r, D1219f, D1876r, D1738f, D2482r, D2338f, and D3069r for *sca4* (~3 065 bp sequence) (Lane, 1991; Roux et al., 1997; Mediannikov et al., 2004; Sekeyova et al., 2001). Additional *ompA* and *ompB* gene fragments were amplified with primers 190-70, 190-602, 190-3588, 190-4406, 190-4338, and 190-5238 for *ompA* (~532 and ~1 650 bp sequences); and 120-M59f, 120-807, 120-607, 120-1497, 120-1378, and 120-2399 for *ompB* (~1 565 bp sequence) (Regnery et al., 1991; Fournier et al., 1998; Roux and Raoult, 2000). PCR products were purified, sequenced, and assembled as stated above. For each gene, a sequence homology search was performed against the NCBI database using BLAST.

For phylogenetic analyses, sequences obtained in this study and those of described species of *Rickettsia* were aligned with GUIDANCE (Penn et al., 2010), using MAFFT algorithm, and a bootstrap of 100 repeats. From the resulting alignments, no columns or sequences were removed, and both ends of the alignments were trimmed with MEGA 5 (Tamura et al., 2011). From these trimmed alignments, the models of nucleotide substitutions were selected using jModelTest (Darriba et al., 2012; Guindon and Gascuel, 2003). The GTR + G model was used for the *gltA*, *ompB*, *sca4* genes, and both trees of the *ompA* gene, the HKY + I model for the *rrs* gene, and the GTR + G model for a concatenated tree using *gltA*, *ompB*, *rrs,* and *sca4* gene fragments. The phylogenetic trees were constructed using a Bayesian MCMC phylogenetic reconstruction with Mr Bayes 3.2 software (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). For constructing the trees, 4 chains were run, with a temperature of 0.2, for a maximum of 2,000,000 generations, discarding the initial 25%, and the standard deviation (SD) of split frequencies at the end of the runs were below 0.01 in all cases. Final editions of the trees were made using MEGA 5.

3. Results

Morphological characteristics placed tick larvae in the genus *Ornithodoros*. The partial sequence of the 16S mitochondrial rDNA gene confirmed the species as *Ornithodoros knoxjonesi* (KU663027).

Both ticks analyzed by PCR to detect *Rickettsia* spp. tested positive for the four genes (*gltA*, *ompA*, *ompB*, *htrA*). For all genes, sequences were identical between the two ticks, confirming that

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