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Research

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Candidatus Rickettsia senegalensis in Cat Fleas (Siphonaptera: Pulicidae) Collected from Dogs and Cats in Cauca, Colombia

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Abstract

Rickettsia typhi and *Rickettsia felis* are flea-transmitted pathogens. They are important causes of acute febrile illness throughout the world. We, therefore, sought to identify the rickettsial species present in the fleas of dogs and cats in the department of Cauca, Colombia. In this study, we collected 1,242 fleas from 132 dogs and 43 fleas from 11 cats. All fleas were morphologically identified as *Ctenocephalides felis* (Bouché) adults and organized in pools for DNA extraction (234 pools from dogs and 11 from cats). The *gltA* gene from rickettsiae was targeted for screening amplification using conventional PCR. In total, 144 of the 245 pools (58.7%) were positive. The positive samples were then processed for the amplification of the *17KDa* gene (144/144; 100% positive) and *sca5* gene (140/144; 97.2% positive). In addition, restriction enzyme length polymorphism analysis using *Nla*IV on the amplified product of the *sca5* gene demonstrated several organisms: 21/140 (15%) were *R. felis*, 118/140 (84.3%) were *Rickettsia asemboensis,* and 1/140 (0.7%) were *Candidatus* Rickettsia senegalensis. Subsequent sequencing confirmed *Candidatus* Rickettsia senegalensis in *C. felis* collected from dogs the first reported from Colombia.

Key words: *Rickettsia asemboensis*, *Rickettsia* spp, *Rickettsia senegalensis*, *Ctenocephalides felis*, flea-borne rickettsioses

Rickettsiae are obligate intracellular gram-negative Alphaproteobacteria that can invade the endothelial cells that line the blood vessels of some vertebrates, as well as the midgut cells of lice, fleas, mites, and ticks (Fang et al. 2017). Members of the genus *Rickettsia* are recognized for their unique relationship with their vector (Reif and Macaluso 2009). In the case of species transmitted by fleas, *Rickettsia typhi* has been described as the causative agent of murine typhus in humans and is transmitted by the oriental rat flea *Xenopsylla cheopis* (Rothschild; Billeter et al. 2016). *Rickettsia felis*, described in the early 1990s (Adams et al. 1990) as a human pathogen (Boostrom et al. 2002), causes flea-borne spotted fever and infects the cat flea *Ctenocephalides felis* (Bouché; Legendre and Macaluso 2017).

During the last decade, different authors have reported *R. felis*-like organisms (RFLOs) in distinct arthropods (Brown and Macaluso 2016), including cat fleas, based on multilocus sequence typing (MLST). The number of species identified as RFLOs has recently increased due to a broader application of molecular methods to various collections of arthropods from different regions. The characteristics of similarity and genetic homology are: 16S rRNA (*rrs*; ≥99.8 %) gene and four protein-coding genes, the *gltA* (≥99.9 %), *ompA* (≥98.8 %), and *sca5* (≥99.2 %) genes and gene D (≥99.3 %) to existing rickettsial species (Fournier et al. 2003).

The species recently proposed as RFLOs are *Rickettsia asemboensis* (Maina et al. 2016), reported in Kenya and Brazil (Silva et al. 2017), and *Candidatus* Rickettsia senegalensis, described in Senegal (Mediannikov et al. 2015; Hornok et al. 2018). Until the present, the pathogenicity of these organisms is not clearly established.

In Colombia, the presence of *R. felis* in fleas in the department of Caldas has been reported (Hidalgo et al. 2013; Ramírez-Hernández et al. 2013), a probable case of flea-borne spotted fever in the department of Cundinamarca has been described (Faccini-Martínez et al. 2013), and *R. felis* has been identified in ticks from Villeta, Cundinamarca (Faccini-Martínez et al. 2016). The aim of this work

was to understand the distribution of flea-borne rickettsiae in the department of Cauca, Colombia.

Materials and Methods

The fleas were collected manually from cats and dogs in four municipalities in the department of Cauca (Fig. 1). The fleas were removed from these animals after consent signed by their owners, following ethical regulations. The fleas from each host were placed directly into 96% ethanol, maintained at room temperature, and transported to the laboratory where they were classified taxonomically using a standard taxonomic key (Linardi and Santos 2012).

The fleas were organized in pools of adult specimens based on the species and host. DNA was extracted with the QIAamp DNA mini kit (Qiagen, Hilden, Germany) modified with an overnight digestion in DNAzol at 56°C and stored at 4°C until being used in PCR amplifications. DNA quality was tested for all pools with conventional PCR for β-*actin* using the primers Actin-FWD and Actin-REV (Table 1). Quantitative real-time PCR was used for the screening of *Rickettsia* spp. (PowerUPSYBRGreen Master Mix Thermo Fisher Scientific, Vantaa, Finland) by amplifying a 147-bp conserved region of the rickettsial citrate synthase gene (*gltA*) using the primers CS-5/CS-6 (Labruna et al. 2004; Table 1); Positive samples from screening were subjected to two different PCR protocols. Amplified products were then used for RFLP analysis to discriminate between various flea-borne rickettsiae. The first PCR protocol was based on amplifying a 434-bp fragment of a portion of the gene encoding the 17kDa antigen of *Rickettsia* spp. (17kDa antigen gene) (Webb et al. 1990) with the primers 17kd1 and 17kd2 by conventional PCR (Table 1); discrimination between *R. typhi* from other flea-borne rickettsiae was performed with the restriction enzyme *Xba*I (New England Biolabs) according to the manufacturer's instructions for identifying *R. typhi* (two bands of 216 and 218 bp) and *R. felis, R. asemboensis,* or *Candidatus* Rickettsia senegalensis (one band of 434 bp; Schriefer et al. 1994). The second was the amplification of a 812-bp fragment of the outer membrane protein B gene (*sca5*) with the primers 120.2788 and 120.3599 (Table 1), and digestion with the restriction enzyme *Nla*IV (New England Biolabs) to distinguish between *R. felis* (one band of 812 bp), *R. asemboensis* (two bands of 490 and 322 base pairs bp), and *Candidatus* Rickettsia senegalensis (two bands of 682 and 130 bp; Blanton et al. 2019). The digested products were electrophoresed on agarose gel at 2% at 100V for 45 min, stained with SYBR safe (Invitrogen) using manufacturer's instructions, and visualized with a UV transilluminator. The samples demonstrating a restriction pattern different from the control DNA

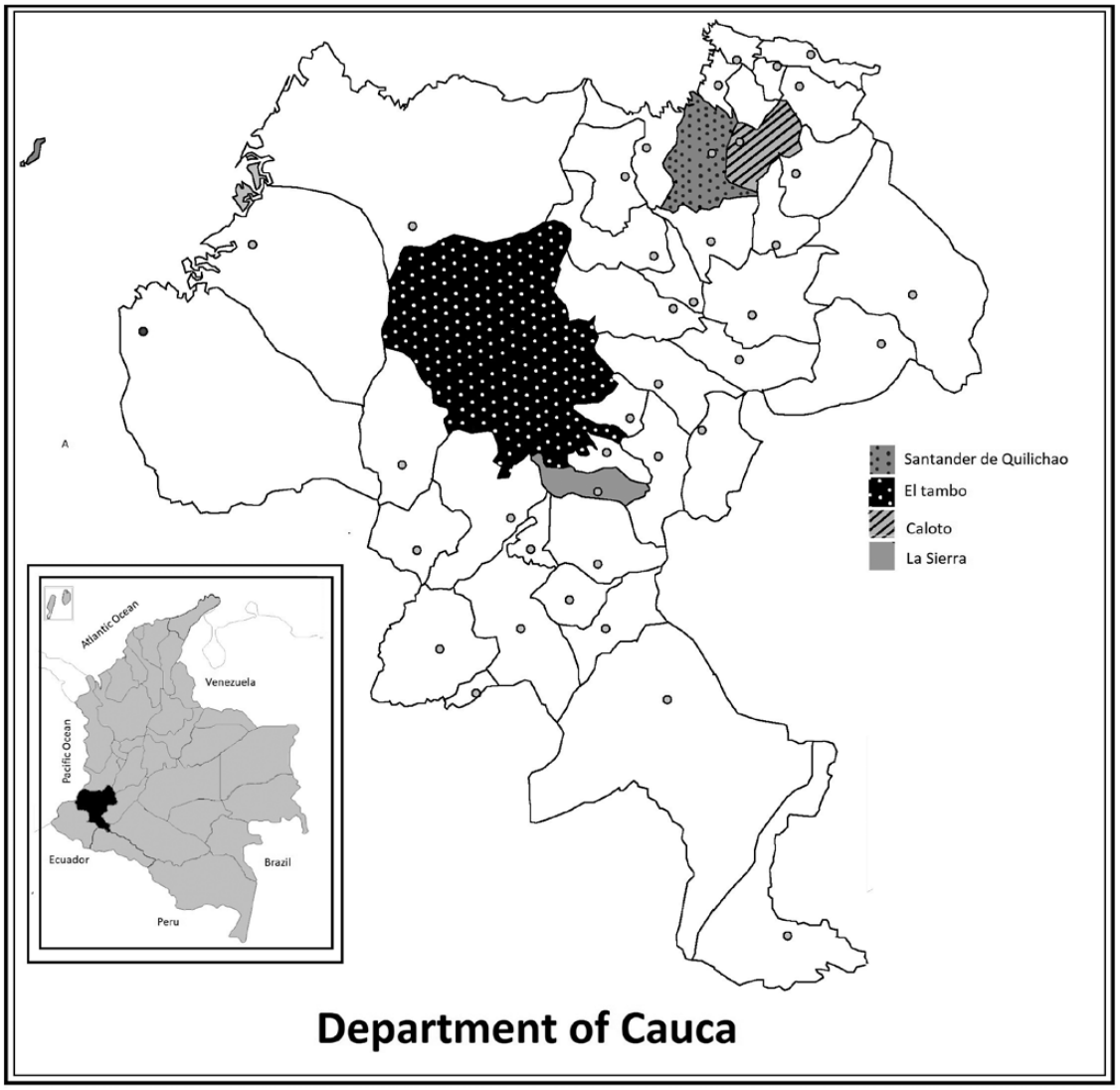

Fig. 1. Map of department of Cauca and municipalities included in the study.

possessed by our laboratory (*R. felis* and *R. typhi*) and the samples that failed to amplify for the *sca5* gene were bidirectionally sequenced using a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were edited using BioEdit v7.0.5.3 (http://www.mbio.ncsu.edu/BioEdit; Hall 1999), aligned with the nucleotide BLASTn program provided by the National Center for Biotechnology Information (NCBI), and compared using the deposited reference sequences in the GenBank (https://blast.ncbi.nlm.nih.gov) after alignment using the Clustal algorithm (Larkin et al. 2007). A phylogenetic analysis was performed using the maximum likelihood (ML) method based on the Kimura 2-parameter model (Kimura 1980), and 1,000 bootstrap replicates were performed using the complete deletion option and the Close-Neighbor-Interchange algorithm of the MEGA software, Version 6 (Tamura et al. 2013). Genomic DNA of *Rickettsia slovaca* was used as a positive control, and molecular grade water was used as a negative control in all PCRs.

Results

In total, 1,285 fleas were collected (1,242 fleas from 132 dogs and 43 fleas from 11 cats). All fleas were morphologically identified as *C. felis* adults. The fleas were analyzed in 245 pools that included one to five fleas (234 pools from dogs and 11 from cats). All samples had successful amplification for the β-*actin* gene; 144 of the 245 pools (58.7%) were positive for the screening amplification targeting *gltA* from rickettsiae. Only the positive samples by *gltA* gene were processed for the amplification of 17kDa antigen gene. Of these specimens, 144/144 (100%) were positive. Digestion with the *Xba*I enzyme revealed that all the analyzed samples were identified as *R. felis*, *R. asembonensis, or Candidatus* Rickettsia senegalensis (Fig. 2). These samples were then analyzed by amplifying the *sca5* gene. Successful *sca5* amplification was achieved in 140/144 (97.2%). Digestion with the *Nla*IV enzyme revealed the following: 21/140 (15%) *R. felis*, 118/140 (84.3%) *R. asemboensis*, and 1/140 (0.7%) *Candidatus* Rickettsia senegalensis (Fig. 3). The four samples that did not amplify the *sca5* gene were sequenced by the 17kDa antigen gene and identified as: 2/4 *R. asemboensis*, 1/4 *R. felis,* and 1/4 the sequence obtained could not be analyzed. The final results are: 22/143 (15.4%) *R. felis*, 120/143 (83.9%) *R. asemboensis*, and 1/143 (0.7%) *Candidatus* Rickettsia senegalensis (Supp Fig. 1 [online only]). Table 2 shows the results of minimum infection rate by municipalities for each of the genes analyzed.

The sample identified by RFLP as *Candidatus* Rickettsia senegalensis was confirmed by sequencing using *gltA* and *sca5* amplicons, obtaining a 99% identity with the strain's sequences KT304219.1 and KF666470.1 deposited in the GenBank. Sequencing of the amplification products of *gltA* and *sca5* genes confirm the identity of the bacteria with *Rickettisia* genus, and molecular phylogenetic analysis of these sequences based on the ML method suggest that the sequences obtained from this flea pool corresponded to *Candidatus* Rickettsia senegalensis demonstrated in the sequence obtained in this work (code: 127; Fig. 4). For the *gltA* phylogenetic tree, 401 nucleotides were analyzed of the amplified product with the primers CS78 and CS-323 (Labruna et al. 2004) and, for the *ompB* phylogenetic tree, 812 nucleotides were analyzed of the amplified product with the primers 120.2788 and 120.3599 (Roux and Raoult 2000). The obtained sequences were deposited in the GenBank with the assession numbers MK548197 and MK548198.

Discussion

This article presents the first report of an organism with homology similar to *Candidatus* Rickettsia senegalensis in Colombia, obtaining 99.8% identity with *gltA* and *sca5* gene sequences previously deposited in GenBank (Mediannikov et al. 2015). This rickettsial species has only been reported in Africa (Mediannikov et al. 2015), Asia (Hornok et al. 2018), India (Khan et al. 2016), the United States

Table 1. Primer sequences for *Rickettsia* genus and species-specific PCRs

Primer name	Gene target	Sequence (5'-3')	References
Actin-FWD	β-*actin*	CGGAACCGCTCATTGCC	(Du Breuil et al. 1993)
Actin-REV	β-*actin*	GCTCACTCAGTGTGGCAAAG	
CS-5	*gltA*	GAGAGAAAATTATATCCAAATGTTGAT	(Labruna et al. 2004)
CS-6	*gltA*	AGGGTCTTCGTGCATTTCTT	
120–3599	*sca5*	TACTTCCGGTTACAGCAAAGT	(Roux and Raoult 2000)
120–2788	*sca5*	AAACAATAATCAAGGTACTGT	
17kDA1	*htrA*	GCTCTTGCAACTTCTATGTT	(Webb et al. 1990
17kDA2	*htrA*	CATTGTTCGTCAGGTTGGCG	

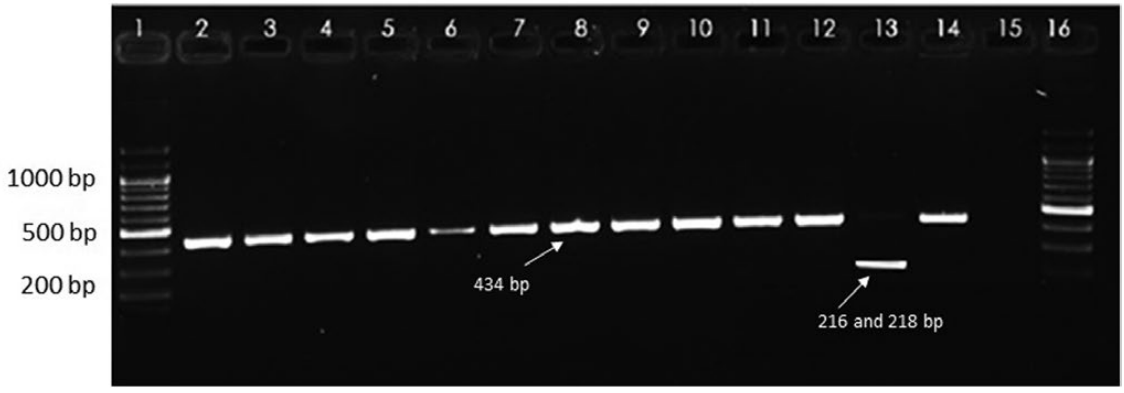

Fig. 2. Comparative restriction enzyme digestion with *Xba*I of 17kDa antigen gene PCR products. MP, ladder 100 bp; lines 1–6, cat flea pools (identified as *R. felis*, *R. asemboensis* or *Candidatus* Rickettsia senegalensis); 7, *R. typhi*; 8, *R. felis*; and 9, negative control.

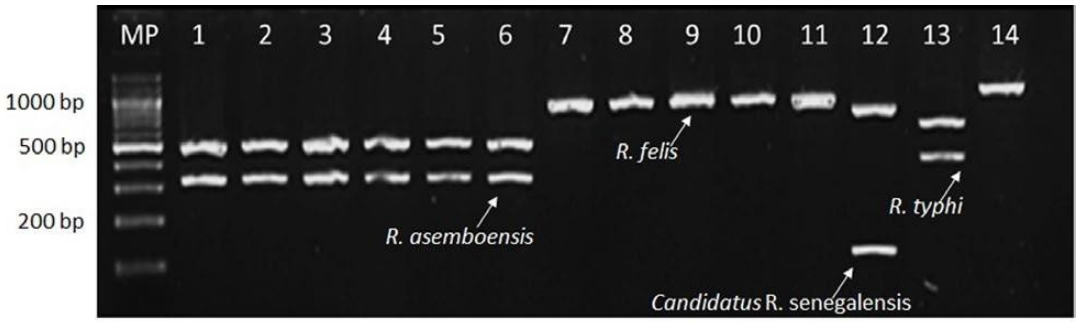

Fig. 3. Comparative restriction enzyme digestion with *Nla*IV of *sca5* gene PCR products. 1, ladder 100 bp; 2–7, cat flea pools identified as *R. asemboensis*; 8–12 and 14–18, cat flea pools identified as *R. felis*; 13, cat flea pool identified as *Candidatus* Rickettsia senegalensis; 19, control *R. typhi*; and 20, control *R. felis*

Table 2. Positive flea samples for *Rickettsia* DNA, collected between August and November 2017, in four municipalities from Cauca Department, Colombia

Municipality	Flea samples[a]					
	gltA		*sca5*		*htrA*	
	Dogs	Cats	Dogs	Cats	Dogs	Cats
La Sierra	4/45 (0.1)	1/5 (0.2)	4/19 (0.2)	0/5 (0.0)	4/19 (0.1)	1/5 (0.2)
El Tambo	76/360 (0.2)	7/25 (0.3)	76/318 (0.2)	7/25 (0.3)	74/318 (0.2)	7/25 (0.3)
Caloto	40/285 (0.1)	1/3 (0.3)	40/227 (0.2)	1/3 (0.3)	39/227 (0.2)	1/3 (0.3)
Santander de Quilichao	15/550 (0.0)	0/10 (0.0)	15/86 (0.2)	—	15/86 (0.2)	—
Total	135/1,240 (0.1)	9/43 (0.2)	135/650 (0.2)	8/33 (0.2)	132/582 (0.6)	9/33 (0.3)

[a]Data are presented as no. of positive pools/total number of fleas tested (MIR).

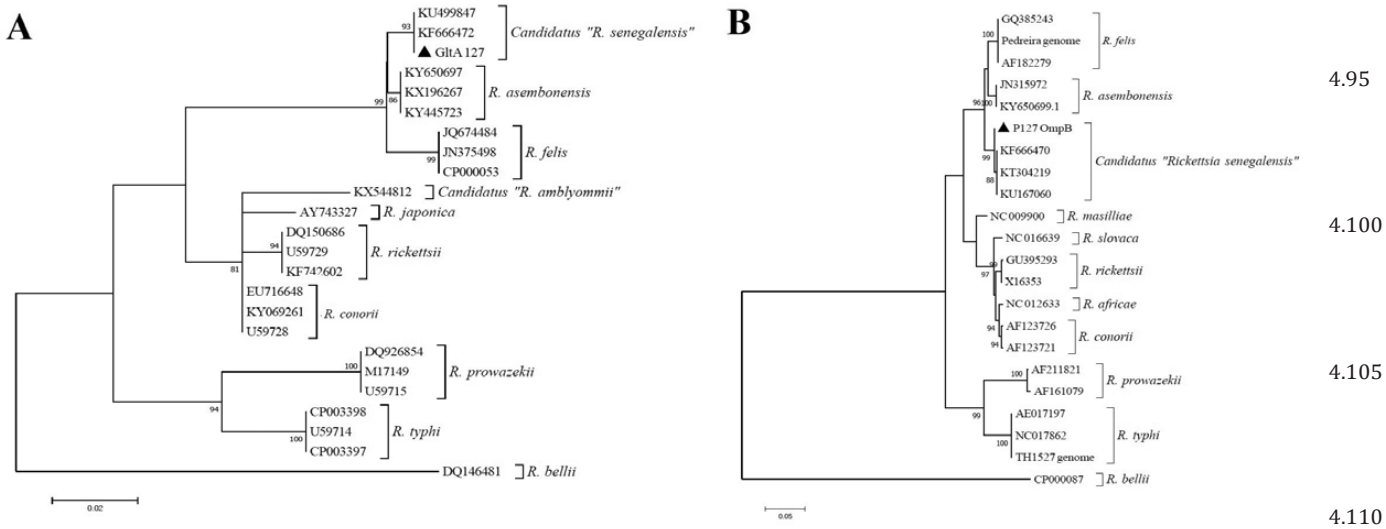

Fig. 4. Phylogenetic analysis via maximum likelihood (ML) analyses of the (A) *glt*A gene of *Rickettsia* spp. and (B) *sca5* genes of *Rickettsia* spp. The tree with the highest log likelihood is shown and is drawn to scale, with branch lengths representing the number of substitutions per site. The sequence retrieved in this study is indicated by black triangles followed by the code name. The GenBank numbers from the reference sequences are indicated. The corresponding *Rickettsia* spp. are listed to the right of each branch.

(Noden et al. 2017), and recently in Chile (Cevidanes et al. 2018). The majority of flea pools collected on dogs and cats from rural areas of Cauca department were positive for different *Rickettsia* species (*R. felis*: 15%, *R. asemboensis*: 84.3%, *Candidatus* Rickettsia senegalensis: 0.7%); however, the analysis of pooled samples prevents us from knowing the exact prevalence in the flea population.

Although the zoonotic capacity of the species found in this study is not yet known and has been hypothesized as a possible commensal (Blanton et al. 2016), it should be noted that *R. asemboensis* was found in isolates of patients with acute febrile syndrome in Peru (Palacios-Salvatierra et al. 2018). Therefore, it is important to study these new species to describe better their ecoepidemiological role and the possibility of being pathogenic to humans.

Ctenocephalides felis was the only flea species captured in the present study, both in dogs and cats. These results coincide with what has been reported in Australia, where this species is more common than *Ctenocephalides canis* (Chandra et al. 2017). This was also demonstrated from a study conducted in Spain, where the

fleas predominantly infesting dogs were *C. felis* (81.7%), followed by *C. canis* (11.4%) and *Pulex irritans* (6, 9%) (Gálvez et al. 2017).

The presence of *C. felis* on dogs and cats demonstrates that it is a multispecies parasite that can serve as a vector for rickettsiae. Furthermore, it represents a risk of human exposure due to the close contact of domestic animals with people (Parola 2011). Moving forward, measures of flea prevention and control, studies to define the pathogenic nature of these species, and the role these rickettsiae play in the cause of acute febrile illness in this region are desired.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

Supplement Fig. 1. Workflow of the experiments and results obtained.

Acknowledgments

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