



A prevalent alpha-proteobacterium *Paracoccus* sp. in a population of the Cayenne ticks (*Amblyomma cajennense*) from Rio de Janeiro, Brazil

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Abstract

As Rocky Mountain Spotted Fever is the most common tick-borne disease in South America, the presence of *Rickettsia* sp. in *Amblyomma* ticks is a possible indication of its endemicity in certain geographic regions. In the present work, bacterial DNA sequences related to *Rickettsia amblyommii* genes in *A. dubitatum* ticks, collected in the Brazilian state of Mato Grosso, were discovered. Simultaneously, *Paracoccus* sp. was detected in approximately 77% of *A. cajennense* specimens collected in Rio de Janeiro, Brazil. This is the first report of *Paracoccus* sp. infection in a specific tick population, and raises the possibility of these bacteria being maintained and/or transmitted by ticks. Whether *Paracoccus* sp. represents another group of pathogenic Rhodobacteraceae or simply plays a role in *A. cajennense* physiology, is unknown. The data also demonstrate that the rickettsial 16S rRNA specific primers used for *Rickettsia* spp. screening can also detect *Paracoccus* alpha-proteobacteria infection in biological samples. Hence, a PCR-RFLP strategy is presented to distinguish between these two groups of bacteria.

Key words: *Paracoccus*, *Rickettsia amblyommii*, *Amblyomma*, ixodidae ticks.

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Ticks are blood-feeding arthropods harboring several associated bacteria, viruses, fungi and protozoans. Although some of the bacteria are classified as either symbionts, or human pathogens, the biological functions of most of these, only recently described prokaryotes, are still unknown (Niebylski *et al.*, 1997; Noda *et al.*, 1997; Marin and Schmidtman, 1998; Marquez *et al.*, 1998; Yparraquirre *et al.*, 2007; Stromdhal *et al.*, 2008; Machado-Ferreira *et al.*, 2009, 2011). In South America, *Amblyomma cajennense* is considered the most important tick species, representing the main vector of the Rhodobacteraceae *Rickettsia rickettsii*, the etiological agent of Rocky Mountain Spotted Fever (Sexton *et al.*, 1993; Lemos 2000; Galvão *et al.*, 2003; Guedes *et al.*, 2005). *A. cajennense* may also harbor several other bacteria, such as *Coxiella*, *Francisella* and other *Rickettsia* species, which may represent new agents of human diseases or symbionts (Sexton *et al.*, 1993; Parola *et al.*, 1998; Machado-Ferreira *et al.*, 2009, 2011).

Emerging infectious disease (EID) agents are comprised of pathogens that recently entered human popula-

tions, most of these with still unknown reservoirs or associated arthropod vectors (Salyers and Whitt, 2002). In this scenario, the CDC Group EO-2 *Paracoccus yeiii*, recently isolated from patients in the US, Canada and Europe, could be classified as another Rhodobacteraceae EID agent, with a currently unidentified source or transmission route (Danešvar *et al.*, 2003; Funke *et al.*, 2004).

In the present study, an attempt was made to detect *Rickettsia* spp. in *Amblyomma* ticks, collected in the Brazilian states of Rio de Janeiro, Minas Gerais and Mato Grosso. We showed that the use of 16S rRNA specific primers for screening *Rickettsia* DNA in biological samples, also proved to be functional in detecting tick-associated *Paracoccus* sp.. This species, by being highly prevalent in *A. cajennense* ticks collected in a sampling site in Rio de Janeiro, possibly represents another group of tick-borne alpha-proteobacteria.

A total of 213 actively feeding adult *A. cajennense* and 20 *A. dubitatum* ticks were collected from horses and a domestic dog in the cities of Sambaetiba, Rio do Ouro and Três Rios (Rio de Janeiro), Pouso Alto (Minas Gerais) and São José do Xingu (Amazonia region of Mato Grosso) between May and November, 2006 (Table 1). After washing engorged females four times in 70% (v/v) ethanol, individuals and pools of ten ticks were snap-frozen in liquid nitro-

Table 1 - Total number of ticks analyzed for *Rickettsia* sp. infection.

Sampling area ¹	Host	Tick species	No. ticks analyzed ²	Minimum infection rate ³
Sambaetiba (RJ)	Horse	<i>A. cajennense</i>	67	0
Rio do Ouro (RJ)	Horse	<i>A. cajennense</i>	12	0
Três Rios (RJ)	Horse	<i>A. cajennense</i>	49	38/49 ⁴
Pouso Alto (MG)	Horse	<i>A. cajennense</i>	85	0
São Jose do Xingu (MT)	Dog	<i>A. dubitatum</i>	20 (2 X 10)	1/20 ⁵

¹RJ, state of Rio de Janeiro; MG, state of Minas Gerais; MT, state of Mato Grosso.

²Most of the ticks were individually analyzed. Samples analyzed by pools are indicated in the parentheses (No. of pools X pool size).

³The minimum infection rate was obtained by assuming that only one tick was infected.

^{4,5}Minimum number of *Paracoccus* sp. ⁴or *Rickettsia* sp. ⁵positive ticks / total number of ticks analyzed.

gen. After homogenization in 1 mL of a lysis solution (sucrose 5%, SDS 1%, 100 mM Tris pH 7.5, 600 mM NaCl and 100 mM EDTA), samples were incubated at 56 °C for 3 h with 0.4 mg/mL of proteinase K and 0.1 mg/mL of ribonuclease A. The resultant DNA was purified by phenol-chloroform extraction and re-suspended in 200 µL of 1 mM Tris pH 7.4 and 0.1 mM EDTA. DNA integrity was analyzed by agarose gel electrophoresis. Tick morphological classification was confirmed by PCR amplification of mitochondrial 16S rRNA genes, using the primer set 16S+1/16S-1 (Black and Piesman, 1994) followed by sequencing and BLAST analyses.

The *Rickettsia* genus-specific primer set fD1-Rc16S.452n (Marquez *et al.*, 1998; Simser *et al.*, 2002) was used to amplify the 16S rRNA gene (16S rDNA). Presumptive *Rickettsia* positive samples were then characterized by PCR amplification by using the Spotted Fever group primer sets, Rr190.70p/Rr190.602n for *ompA* (Regnery *et al.*, 1991; Simser *et al.*, 2002), the *Rickettsia*-specific primer set named Rr120 for *ompB* (Gage *et al.*, 1992; Noda *et al.*, 1997), Rr17.61p/Rr17.491n for *htrA* (Williams *et al.*, 1992; Simser *et al.*, 2002), and RpCS.877p/RpCS.1258n for *gltA* (Regnery *et al.*, 1991; Simser *et al.*, 2002). PCR temperature/time cycling conditions were 35 cycles of 94 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min. PCR products from four independent reactions were then merged and directly purified with a GFX PCR DNA and Gel band purification kit (GE Healthcare, Buckinghamshire, UK), according to manufacturer's instructions. In the case of suspect spurious PCR products, bands of expected size were cut from agarose gels and used as PCR templates with the same primer set. Purified PCR products were sequenced using the BigDye Terminator DNA sequencing kit (Applied Biosystems, Foster City, Calif., U.S.A.), and analyzed in a Megabace 1000 DNA sequencer (Amersham Biosciences). Sequences were edited using the SeqMan program (DNASTARinc package for the Windows platform, 1989-1999), and gene identities obtained by BLAST analyses (blastn).

From the 233 ticks analyzed from distinct geographic locations, 94 DNA samples presented the expected amplicon size of ~430 bp for *Rickettsia* spp. 16S rDNA

(Named "Major-94" sample group) (data not shown). However, only one DNA sample (A45), from a pool of 10 *A. dubitatum* ticks collected in São José do Xingu was also PCR positive for the *Rickettsia* genes *ompA* (~530 bp), *ompB* (~500 bp), *gltA* (~380 bp) and *htrA* (~430 bp) (data not shown). Sequence analyses confirmed that sample A45 contained gene sequences corresponding to 16S rDNA (GenBank EF611737), *htrA* (GenBank EF611738) and *ompA* (GenBank Bankit_1476251), these, respectively, being 99%, 98% and 100% identical to the related genes of *Rickettsia amblyommii* (GenBank U11021; DQ517291 and AY062007). Phylogenetic analysis with 16S rDNA gene and *htrA* sequences clearly included the sample A45, denoted "*Rickettsia* sp. A45," within the *Rickettsia* Spotted-Fever group (Figure 1A,B). The *ompA* sequences gave an even better resolution with the *Rickettsia* Spotted-Fever group. In this tree the close relationship between *Rickettsia* sp. A45 and *R. amblyommii* was evident, with a 99% bootstrap support (Figure 1C).

In the Major-94 group, DNA samples from the 55 *A. cajennense* ticks collected in Sambaetiba (RJ) yielded numerous spurious PCR bands. According to initial-replicon analysis, re-amplification and sequencing, they were not identical to either *Rickettsia* sp., or to any other bacterial DNA. From these apparently positive samples, 17 tick-bulk DNA samples were also PCR analyzed, in an attempt to detect *ompA*, *ompB*, *htrA* and *gltA* genes. No amplification was obtained, confirming that they were in fact free of *Rickettsia* spp. (data not shown). Four randomly selected DNA samples of *A. cajennense* collected at Três Rios (RJ), and pertaining to the same group, were also sequenced and analyzed. Although these nucleotide sequences (GenBank EF531228) were all identical, there was no similarity to any *Rickettsia* spp. sequence. Even so, there was a close relationship to soil and invertebrate-associated alpha-proteobacteria *Paracoccus* spp., the latter mostly of the *Paracoccus* sp. SA5 strain (GenBank AY864654) and the soil *P. alcaliphilus* KTC002 (GenBank AJ294415), with 99% and 98% identity, respectively (Figure 1A). Thus, there is every indication that an *A. cajennense* population at Três Rios is infected with a specific *Paracoccus* sp. population, denoted here as "*Paracoccus* sp.-Cayenne". This bac-

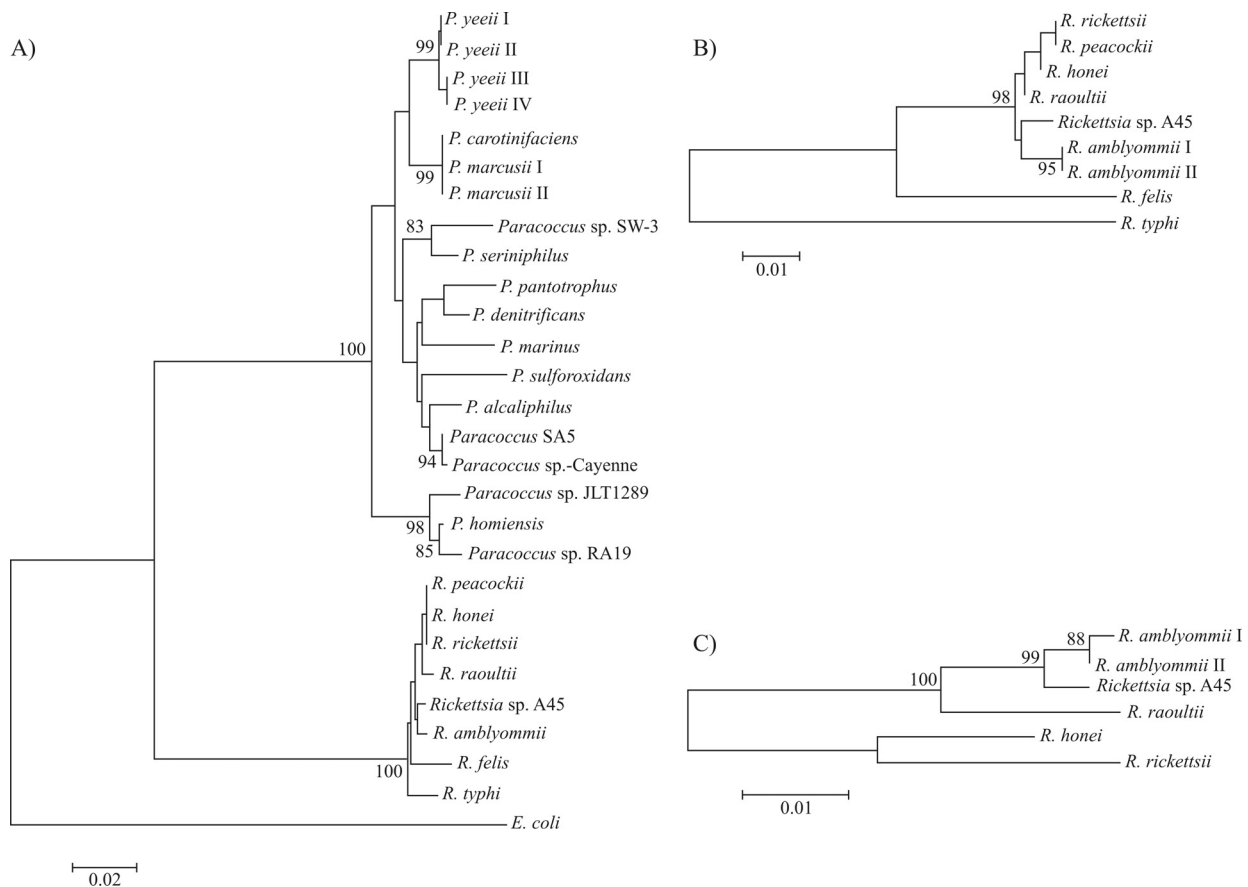


Figure 1 - Phylogenetic inferences by Neighbour-Joining (NJ) with Kimura 2-parameter. GenBank accession numbers are presented in parenthesis. A) 16S rDNA gene sequence alignment of *Paracoccus* sp.-Cayenne and *Rickettsia* sp. A45, *P. yeeii* I (AY014178), *P. yeeii* II (DQ857285), *P. yeeii* III (AY014179), *P. yeeii* IV (AY014179), *P. carotinifaciens* (AB006899), *P. marcusii* I (DQ298023), *P. marcusii* II (DQ298022), *Paracoccus* sp. SW-3 (FJ593906), *P. seriniphilus* (AJ428275), *P. pantotrophus* (AB098590), *P. denitrificans* (CP000490), *P. marinus* (AB681212), *P. sulforoxidans* (JQ291588), *P. alcaliphilus* (AJ294415), *Paracoccus* SA5 (AY864654), *Paracoccus* sp. JLT1289 (EU650196), *P. homiensis* (NR_043733), *Paracoccus* sp. RA19 (AJ507806), *R. peacockii* (DQ062433), *R. honei* (U17645), *R. rickettsii* (U11021), *R. raoultii* (DQ365809), *R. amblyommii* I (U11012), *R. felis* (CP000053), *R. typhi* (AE017197) and *E. coli* (AY84014). B) *htrA* gene sequence alignment of *Rickettsia* sp. A45, *R. rickettsii* (DQ176856), *R. peacockii* (AF260571), *R. honei* (AF060704), *R. raoultii* (EF392727), *R. amblyommii* I (DQ517291), *R. amblyommii* II (AY375162), *R. felis* (CP000053) and *R. typhi* (AE017197). C) *ompA* sequence alignment of *Rickettsia* sp. A45, *R. amblyommii* I (AY062007), *R. amblyommii* II (EF194096), *R. raoultii* (DQ365799), *R. honei* (AF018075) and *R. rickettsii* (DQ002504). Sequences were aligned using the CLUSTAL W 2.0 program, and phylogenetic inferences obtained using MEGA 5 software. Internal node supports were calculated using bootstrap with 1000 replicas. Bootstrap values below 80% are not presented.

terial population shows 96% 16S rRNA gene identity to the clinical *P. yeeii* strain G3060 (GenBank AY014179). PCR products from a total of 38 Três Rios tick samples, positive for the ~430 bp 16S rDNA amplicon were subjected to additional endonuclease digestion. A RFLP strategy was designed to specifically assign these amplicons to either the *Rickettsia* or the *Paracoccus* 16S rDNA group. After analyzing the 16S rRNA gene sequences presented in Figure 1A, we observed that every *Rickettsia* 16S rRNA gene sequence included in the primer set fD1-Rc16S.452n amplicon generated the same *Hinf*I digestion profile, with the presence of 117 bp, 124 bp and 195 bp bands, whereas all the *Paracoccus* sequences generated a profile with bands of 25 bp, 100 bp and 297 bp, this including *P. sp.-Cayenne*. All 38 samples from Três Rios showed the typical restriction enzyme digestion profile expected for the *Paracoccus* group (Figure 2). The 14 Três Rios Cay-

enne-tick DNA samples were all negative for additional PCR reactions when using primer sets for *ompA*, *ompB*, *gltA* and *htrA* genes (data not shown), thereby confirming the absence of *Rickettsia* sp.

The genus *Paracoccus* comprises metabolically versatile bacteria, which are either aquatic or terrestrial free-living microbes or are associated with other organisms, such as plants, humans and invertebrates, including insects (Baker *et al.*, 1998; Baj, 2000; Daneshvar *et al.*, 2003; Funke *et al.*, 2004; Amarasekare *et al.*, 2008; Huang *et al.*, 2012; Kämpfer *et al.*, 2012; Singh *et al.*, 2012). Interestingly, even though *Paracoccus* spp. have never been identified in ticks, the application of primers designed for *Rickettsia* 16S rDNA, followed by sequence analysis and RFLP, revealed the presence of *P. sp.-Cayenne* in ~77% of the cayenne ticks collected at Três Rios (Table 1). Phylogenetic inference, using partial 16S rDNA sequences,

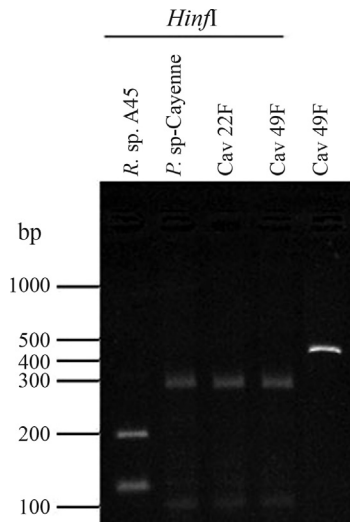


Figure 2 - Agarose gel of 16S rDNA PCR products digested with *Hin*I. PCR reactions were achieved using the *Rickettsia* spp. specific primer set, as described in the text. Lanes include amplicon digestions for *Rickettsia* sp. A45 (Lane 1) and *Paracoccus* sp-Cayenne (Lane 2), as well as the tick-total DNA samples Cav22F and Cav49F (Lanes 3-4). The non-digested PCR product for Cav49F was run in parallel (Lane 5). The molecular marker is presented in base pairs (bp). The expected bands for the *Rickettsia* sample include the 195 bp, 117 bp and 124 bp bands, the last two appearing as a single band in the gels. The *Paracoccus* sp-Cayenne expected profile includes the bands of 297 bp and 100 bp, as well as the faint band of 25 bp, not observed in the gels.

places *P. sp-Cayenne* into a soil-associated *Paracoccus* group, closely related to the *Paracoccus* sp. SA5 strain (Figure 1A). Due to the proximity of *P. sp-Cayenne* and free-living soil isolated *Paracoccus*, the possibility of an accidental contamination was assessed, in an attempt to detect *Paracoccus* sp-Cayenne in soil samples from the same area at Três Rios. Soil samples were collected from four distinct areas used for horse pasture. A mixture of 7 g of each soil sample with 6 mL of TE was shaken, and after particle decantation, DNA was purified by phenol-chloroform or DNeasy Kit (Qiagen, Valencia, CA) extraction. After PCR reactions using Eubacteria universal primers 27f/1492R (Lane, 1991) and a *Rickettsia* sp. 16S rDNA specific primer set, all samples proved to be positive for Eubacteria, although only one presented positive amplification using the *Rickettsia* primer set. Subsequent sequence analysis of this DNA sample indicated sequence relationship to a soil *Acidobacteria* (data not shown). Nevertheless, this geographically localized analysis does not rule out the soil origin of this tick-associated *Paracoccus* sp.

Over the last two decades, several new members of the *Rickettsia* genus were identified in association with ticks. Most were classified as tick symbionts, while others were considered EID agents (Parola *et al.*, 1998; Fournier *et al.*, 2000; Beninati *et al.*, 2002; Nilsson *et al.*, 2002). Many of the symbionts were further reclassified as pathogenic agents (Fournier *et al.*, 2000; Nilsson *et al.*, 2002). Within this scenario, *R. amblyommii*, which was identified

in *Amblyomma* ticks from Brazil and the United States, could possibly represent an unknown rickettsiosis agent (Stothard and Fuerst, 1995; Labruna *et al.*, 2004; Stromdahl *et al.*, 2008), due to a suggestive association of *R. amblyommii* infections with several cases of a Rocky Mountain spotted-fever-like disease, ascertained by serological assays (Apperson *et al.*, 2008), and the fact that *R. amblyommii* antigens cross-react with antibodies against *R. rickettsii* (Walker *et al.*, 2008). In the present study, the presence of *Rickettsia* sp. A45 DNA in *A. dubitatum* collected in the Amazon region of São Jose do Xingu (MS), was confirmed. Sequence analysis showed that *Rickettsia* sp. A45 is closely related to *R. amblyommii* (Figure 1), which was also recently described in *Amblyomma* ticks collected in the Amazon (Labruna *et al.*, 2004). Although the pathogenic potential of *Rickettsia* sp. A45 was not assessed, *ompA* phylogenetic analysis (Figure 1C) indicated certain similarities to *R. amblyommii*, through the OmpA protein being directly associated with host-cell adherence and rickettsial pathogenicity (Blanc *et al.*, 2005).

Primer sets for the *Rickettsia* genes *ompA*, *ompB*, *htrA*, *gltA* and 16S rDNA have been collectively and successfully applied to diagnose rickettsial infection (Regnery *et al.*, 1991; Fournier *et al.*, 2000; Simser *et al.*, 2002; Pacheco *et al.*, 2007; Phan *et al.*, 2011; Radulovic *et al.*, 2011; Zou *et al.*, 2011). However, our results suggest a limitation in the use of the 16S rDNA primer set fD1-Rc16S, since PCR reactions using these primers also revealed the presence of *P. sp-Cayenne* in approximately 77% of *A. cajennense* ticks collected at Três Rios (Figures 1A and 2). This suggests certain limitations when rickettsial 16S rRNA specific primers are solely used to assess *Rickettsia* infection in a biological sample.

Phylogenetic inference clustered *P. sp-Cayenne* 16S rDNA sequences with soil *Paracoccus* sp. Interestingly, the phylogenetic reconstruction did not keep soil, invertebrate associated, and clinical *Paracoccus* clusters robustly apart (bootstrap values < 80%) (Figure 1A), thereby reflecting an intimate phylogenetic relatedness grouping all these organisms (Baker *et al.*, 1998). A robust *Paracoccus* cluster was observed, keeping the marine group with the isolates *P. sp. JLT1284*, *P. homiensis* and *P. sp. RA19* apart. It is important to note that the human-infectious *P. yeeii* cluster is not only closely related to the insect-associated *Paracoccus marcusii* cluster, but is often detected in patients with leg or foot wounds (Daneshvar *et al.*, 2003; Funke *et al.*, 2004), thus, a possible indication of vector bites. Recently a *P. yeeii* infection, associated with a peritonitis in a habitual horse rider (Wallet *et al.*, 2010), could be correlated with the presence of *P. sp-Cayenne* encountered in horse-fed ticks at Três Rios. Also recently, several uncultured bacteria phylotypes with 16S rRNA gene sequences 99% identical to *P. sp-Cayenne*, were identified as composing the skin microbiota associated with human dermatitis (Grice *et al.*, 2009; Kong *et al.*, 2012) and infecting gastro-

intestinal tissues (Frank *et al.*, 2007) (data not shown). It is not clear whether *P. sp.-Cayenne* represents a potential pathogen, or is capable of competing with, or even exacerbating, other human tick-borne infectious agents. Even though possibly tick symbionts, other recognized symbiotic bacteria in Ixodidae are also potentially pathogenic (Burgdorfer *et al.*, 1973). Although *Paracoccus* sp. was not specifically isolated in this study, this is the first report of *Paracoccus* sp. detection in ticks, thereby indicating that this bacterial group is harbored in Brazilian tick populations. Future studies should focus on isolating this *Paracoccus* sp. and on infected tick metagenomics, to clearly define both its pathogenic potential and/or its role in promoting aspects of *A. cajennense* biophysiology.

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