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Two for the price of one: Co-infection with *Rickettsia bellii* and spotted fever group *Rickettsia* in *Amblyomma* (Acari: Ixodidae) ticks recovered from wild birds in Brazil

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ABSTRACT

The bacterium *Rickettsia bellii* has been detected in 25 species of ticks in the American continents, but its pathogenic potential is considered as undetermined. A possible role for this species in the phenomenon of transovarial exclusion of pathogenic members of the spotted fever group (SFG) of *Rickettsia* has been suggested and co-infections with pathogenic species have been reported infrequently in both North and South America. Traditional methods for the molecular detection of rickettsial agents in ticks focus largely on the identification of sequences found in SFG *Rickettsia*, an approach that may overlook the presence of co-infections with *R. bellii*. Two novel, species-specific polymerase chain reaction (PCR) assays, targeting the genes encoding the surface cell antigen (Sca), autotransporter proteins sca9 and sca14, were developed and validated for the detection of *R. bellii* using 150 *Amblyomma* ticks collected from wild birds in Brazil. Co-infection of *R. bellii* infected ticks was evaluated using a novel PCR assay targeting the *ompA* sequence characteristic of SFG *Rickettsia*. Preliminary species-level identification was achieved by restriction fragment length polymorphism (RFLP) analysis and subsequently confirmed by sequencing of amplicons. Nine out of seventy-three *Amblyomma longirostre* and one of two *Amblyomma calcaratum* ticks were shown to be co-infected with *R. bellii* and *Rickettsia amblyommatis*, while two out of sixty-seven *Amblyomma* sp. haplotype Nazaré ticks were recorded as co-infected with *R. bellii* and the *Rickettsia parkeri*-like bacterium, strain ApPR. Interestingly, our data represent the first records of *R. bellii* in association with *A. calcaratum and Amblyomma* sp. haplotype Nazaré. The novel PCR-RFLP systems reported herein, provide an alternative, rapid and cost-efficient (relative to strategies based on sequencing or real-time PCR), approach to evaluate rickettsial co-infection of ticks, a potentially significant phenomenon that has most likely been underestimated to date.

1. Introduction

The genus *Rickettsia* comprises a diverse group of Gram-negative bacteria characterized by an obligate intracellular lifestyle and the notorious role of some species as arthropod-borne pathogens of man and animals (Diop et al., 2018; Parola et al., 2013). The true extent of human infections with *Rickettsia*, is believed to be underestimated because of factors including incorrect diagnosis owing to similarities in symptomology with other febrile illnesses, lack of knowledge concerning *Rickettsia* among medical professionals and the absence of convenient and accessible diagnostic tests (Faccini-Martínez et al., 2014; Oliveira et al., 2016).

Rickettsia bellii, a member of the ancestral group of Rickettsiae, was reported for the first time in Brazil in 2004 infecting five species of *Amblyomma* ticks (Labruna et al., 2004). It has since been recorded in an additional seven species of *Amblyomma* and in *Ixodes loricatus*, and

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Haemaphysalis juxtakochi ticks (Costa et al., 2017; McIntosh et al., 2015; Parola et al., 2013). Uncertainty, in relation to the pathogenic potential of *R. bellii*, emerged based on the observation that subcutaneous inoculation induced the development of eschars in rabbits and guinea pigs (Ogata et al., 2006), a symptom considered characteristic for a number of rickettsioses (Faccini-Martínez et al., 2014). In addition, serological data from Brazilian studies indicated the capacity to cause natural infections in capybaras (*Hydrochoerus hydrochaeris*) (Pacheco et al., 2007), dogs (Spolidorio et al., 2013) and horses (Vieira et al., 2018), albeit with no evidence to support the use of such animals as amplifier hosts; a strategy employed by pathogenic SFG *Rickettsia*, including *Rickettsia rickettsii* and *Rickettsia conorii* (Parola et al., 2013). Data acquired from observation of naturally infected colonies of *Amblyomma dubitatum* (Sakai et al., 2014) and *I. loricatus* (Horta et al., 2006), demonstrated highly efficient transstadial survival and transovarian transmission, suggesting that *R. bellii* is most likely maintained in nature via those mechanisms.

The term rickettsial exclusion was proposed by Burgdorfer et al. (1981), based on epidemiological observations and experimental evaluation of transovarian transmission, to describe the phenomenon whereby primary infection by one rickettsial agent (*Rickettsia peacockii*) served to impede ovarian infection by a second species (*R. rickettsii*) in *Dermacentor andersoni* ticks in the USA (Burgdorfer et al., 1981). Additional examples of this exclusion effect were subsequently reported for *Rickettsia rhipicephali* by *Rickettsia montanensis* in *Dermacentor variabilis* (Macaluso et al., 2002), and were alluded to for *R. rickettsii* by *R. bellii* in *A. dubitatum* (Sakai et al., 2014). This phenomenon has also been suggested as the most plausible explanation for the apparent exclusion of *Rickettsia parkeri* sensu stricto (s. s.) in populations of adult *A. maculatum* ticks in some states of the USA with a high prevalence of "*Candidatus* Rickettsia andeanae" (Lee et al., 2017; Paddock et al., 2015). In contrast, the absence of such exclusion has been demonstrated in a recently published study by Levin et al. (2018).

Rickettsia bellii has been reported, albeit sporadically, in natural co-infections with other rickettsial agents; *R. rhipicephali* in *D. variabilis* collected in the state of Arkansas, USA (Wikswo et al., 2008), *R. montanensis* and *R. rickettsii* in *D. variabilis* collected in the state of Ohio, USA (Carmichael and Fuerst, 2010), and with *R. parkeri* in *Amblyomma ovale* in Brazil (Szabó et al., 2013). In all three cases, the initial evidence for co-infections was encountered fortuitously based on the observation of dual peaks at multiple base positions throughout sequencing electropherograms, obtained from PCR amplicons of genus-specific gene targets i.e. citrate synthase (Szabó et al., 2013; Wikswo et al., 2008), or the 17-kDa antigen (Carmichael and Fuerst, 2010). Confirmation of the presence of *R. bellii* was subsequently achieved by PCR using primers specific for the citrate synthase gene (*gltA*) of *R. bellii* (Carmichael and Fuerst, 2010; Szabó et al., 2013). In an attempt to facilitate the differential detection of *R. bellii* in ticks, Hecht et al. (2016), reported the development and validation of a real-time PCR assay for the specific detection of *R. bellii* using, once again, the *gltA* gene sequence as the target.

The current work reports the development and evaluation of a novel, rapid and cost-effective approach to investigate the phenomenon of co-infection of ticks with *R. bellii* and SFG rickettsial agents. The application of this strategy to ticks collected from wild birds, captured in different regions of Atlantic rainforest in Brazil, demonstrated that co-infections with *R. bellii* can be conveniently and differentially detected during the first round of molecular screening, reducing the requirement for more expensive techniques including sequencing or real-time PCR.

2. Material and methods

2.1. Development of species-specific PCR assays for R. bellii

Table 1
Primers used in this study.

Target	Primer	Primer Sequence (5´-3´)	Amplicon size (bp)
*sca*3	Forward	CCA CTA TTA ATT TTA ACG GGG CA	**654**
	Reverse	TAC CCG TAC CAC CGC TAA AC	
*sca*8	Forward	ACG ATT GAA AGA GCA GAC GG	**723**
	Reverse	ACC ACC GGA AGT ATG ACC TC	
*sca*9	Forward	CAG CAG CCT TTC ACG TTT CT	**727**
	Reverse	ACT CCA ATG TCA GCA ATA CTA GG	
*sca*14	Forward	CCA CAG AAT TTG CAC CAC GT	**724**
	Reverse	TTT GTT TGA GCA CTC GGT ATT TT	
*htr*A	Forward	GGA ACC AGG CGG TAT GAA TAA	**407**
	Reverse	ACT TGC CAT AGT CCG TCA GG	
*omp*A	Forward	GCT TTA TTC ACC TCA AC	**357**
	Reverse	GCC GGC AGT AAT AGT AAC AG	

The targets chosen for the development of *R. bellii* specific primers were four genes (*sca3*, *sca8*, *sca9* and *sca14*) encoding surface cell antigen (Sca), autotransporter proteins (Sears et al., 2012). Sequences of the full-length genes were obtained from the GenBank (Accession numbers; AY973495, AY973496, AY973497 and AY970512) and primers were designed (Table 1), to generate amplicons of approximately 700 base pairs (bp), using the program Primer3 (Untergasser et al., 2012). Primer specificity was examined *in silico*, via primerBLAST, with comparison to sequences in the nucleotide collection (nt) database. This was deemed necessary since the template DNAs to be examined were obtained from complex environmental samples derived from tick homogenates, many of which contained avian blood and diverse microbiotas. The composition of the PCR master-mixes and the cycling conditions were optimized using three concentrations of MgCl$_2$ (2.0 mM, 2.5 mM and 3.0 mM) and a temperature gradient ranging from 50 °C to 60 °C, using DNA from *R. bellii* (strain Mogi; kindly provided by Dr. Marcelo B. Labruna, Department of Preventative Veterinary and Animal Health, Faculty of Veterinary Medicine, University of São Paulo, Brazil), as the template. The *R. bellii* control DNA was derived from infected Vero cell cultures and as such, it could not be accurately quantified without the use of real-time PCR methods. Thus, to estimate the quantity of rickettsial DNA in the sample we used a limiting dilution approach, whereby dilutions were prepared from 10^{-1} to 10^{-6} in AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0). Determination of the last dilution containing detectable rickettsial DNA was assessed by amplifying a 407-bp fragment of the *htrA* gene, using primers 17KDF (5´-GGA ACC AGG CGG TAT GAA TAA-3´) and 17KDR (5´-ACT TGC CAT AGT CCG TCA GG-3´), as reported by Santolin et al. (2013), employing the master-mix and cycling conditions related by Zeringóta et al. (2017). Thereafter, the sensitivity of the four sets of *R. bellii*-specific primers was compared using the same dilutions of the *R. bellii* DNA as described above, in combination with the optimized reaction conditions (annealing temperature and concentration of MgCl$_2$), of each assay. The primer sets designed to amplify *sca9* and *sca14*, showed sensitivities equivalent to that of the *htrA* assay, while the other two primers sets were 10-fold less sensitive and as such were not examined further. The specificity of the *sca9* and *sca14* assays was assessed using two types of negative controls. Firstly, DNA extracted from a total of 52 *Amblyomma* ticks collected from wild birds and characterized as PCR negative for rickettsial DNA as reported previously (Luz et al., 2017;Zeringóta et al., 2017). The samples used were as follows; *Amblyomma aureolatum* (n = 2), *A. brasiliense* (n = 6), *A. calcaratum* (n = 6), *A. longirostre* (n = 10), *A. naponense* (n = 2), *A. nodosum* (n = 3), *A. ovale* (n = 2), *A. parkeri* (n = 6), *A. sculptum* (n = 4), *A. varium* (n = 1), and *Amblyomma* sp. haplotype Nazaré (n = 10). Secondly, DNA extracted from Vero cell cultures infected with the following species of *Rickettsia*: *R. amblyommatis* (strain AC37), *R. felis* (strain Pedreira), *R. monteiroi* (strain 7 P), *R. parkeri* (strain Atlantic rain forest-A010), *R. rhipicephali* (stain HJ5) and *R. rickettsii* (Taiaçu) (all provided by Dr. Marcelo B. Labruna). Positive controls, included to confirm the ability of the assays to detect *R. bellii* DNA in tick homogenates, comprised DNA extracted from seven *Amblyomma rotundatum* ticks (five females and two nymphs), collected from toads

(*Rhinella marina*) in the Brazilian Amazon and previously characterized as infected with *R. bellii* (Luz et al., 2018). The PCR mixtures (12.5 μL) contained 1.25 μL of Platinum Taq DNA polymerase buffer (Thermo Fisher Scientific, Brazil), 2.5 mM $MgCl_2$, 200 μM dNTPs (Sinapse Biotecnologia, Brazil), 10 pmoles of each primer, 0.5 units of Platinum Taq DNA polymerase and 2 μL of DNA template. The cycling conditions employed were an initial denaturation at 95 °C for 5 min; followed by 45 cycles of denaturation at 95 °C for 20 s, annealing at 52 °C for 20 s, extension at 72 °C for 25 s, with a final extension step at 72 °C for 5 min. Five microliters of PCR products were analysed by gel electrophoresis (1.5% agarose; KASVI, Spain), with confirmation of amplicon sizes achieved via comparison to a DNA molecular weight marker (GeneRuler 100 bp DNA Ladder; Thermo Scientific). The remainder of each reaction was stored at −20 °C for additional molecular analyses as described below.

2.2. DNA extracted from bird ticks characterized as infected with SFG Rickettsia and examined for the presence of rickettsial co-infection

Details of the DNA samples (n = 150), examined in this study for evidence of co-infection are provided in Supplementary data Table S1. In common with the *Rickettsia*-negative ticks detailed above, the selected material was obtained from within the arthropod nucleic acid sample collection, maintained at the Molecular Biology Laboratory, Department of Animal Parasitology, Federal Rural University of Rio de Janeiro (UFRRJ). Details of tick collection and their identification to the species level were as reported in three previous studies investigating bird-tick-*Rickettsia* interactions in four distinct regions of Brazilian Atlantic rainforest; three in the state of Rio de Janeiro (Luz et al., 2017; Santolin et al., 2013) and the fourth in the state of Minas Gerais (Zeringóta et al., 2017). The majority of the ticks (114/150), had been characterized as infected with SFG rickettsial agents in the aforementioned studies. The remaining 36 ticks, collected as a component of the study of Santolin et al. (2013), were previously identified as *A. longirostre* by sequencing of a 460-bp fragment of the mitochondrial sequence encoding 16S rDNA, using the methods reported by Mangold et al. (1998), but they had not been examined for the presence of *Rickettsia* DNA. Each of the 36 ticks were found to be infected with *R. amblyommatis*, as determined using the *ompB*/PCR-RFLP method developed by Santolin et al. (2013), and confirmed via PCR and sequencing of *ompA* amplicons generated with the primers Rr190.70p and Rr190.602n applying the cycling conditions reported in Zeringóta et al. (2017).

The choice of the samples examined (n = 150), specifically *Amblyomma calcaratum* (n = 2)*, Amblyomma longirostre* (n = 73), *Amblyomma parkeri* (n = 8) and *Amblyomma* sp. haplotype Nazaré (n = 67), characterized as infected with *R. amblyommatis* (n = 75), *R. parkeri* (n = 45) and *R. rhipicephali* (n = 30), was essentially random, albeit guided by the relative abundance of each tick species within the nucleic acid sample collection.

2.3. Novel PCR assay for detection of SFG Rickettsia using the ompA gene as a target

The tick DNA samples (n = 12), identified as positive for the presence of both *R. bellii* (using the *sca9* and *sca14* assays) and a SFG rickettsial agent (previously determined via PCR and sequencing of *ompA* amplicons generated with the primers Rr190.70p and Rr190.602n), were examined using a novel PCR assay designed to amplify a 357-bp fragment of the SFG-specific *ompA* gene. This assay was developed to provide a detection system that was comparable, in terms of sensitivity, to the *sca9*, *sca14* and *htrA* assays. The novel assay used the primers ompAdogF (5′-GCT TTA TTC ACC ACC TCA AC-3′) and ompAdogR (5′-GCC GGC AGT AAT AGT AAC AG-3′), designed to generate a product of 357-bp for all the SFG *Rickettsia* reported to date infecting Brazilian bird ticks (Luz et al., 2017) and also for *R. rickettsii*. *In silico* analysis indicated that the new primers would not amplify the *ompA* sequences of *R. felis* or *Rickettsia asembonensis*, two species associated predominately with fleas, but that have been infrequently detected in Brazilian ticks (Bitencourth et al., 2017; Dall'Agnol et al., 2017). The composition of the master-mix and the cycling conditions were the same as those used for the *sca9* assay, except for the extension time which was reduced to 15 s. This assay was assessed for specificity, defined as amplification of the SFG (*R. amblyommatis*, *R. monteiroi*, *R. parkeri*, *R. rhipicephali* and *R. rickettsii*) positive control DNAs, but with no amplification of the *R. bellii* DNA and for sensitivity, determined via the limiting dilution approach, using the *htrA* assay as the standard to determine the last dilution containing amplifiable rickettsial DNA.

2.4. Identification of amplicons

Owing to the complex nature of the DNA samples used in this study, it was considered necessary to perform post-amplification analysis of all presumptive *R. bellii* amplicons by nucleotide sequencing using the protocols reported in Luz et al. (2017). In addition, the possibility of using PCR-Restriction fragment length polymorphism (PCR-RFLP), as an alternative, rapid and cost-efficient means to confirm the identity of amplicons was evaluated *in silico* using the free, online program NEBcutter V2.0 (Vincze, 2003). Based on the results of those analyses, the enzymes *Alu*I (for *sca9*), *Eco*RV (for *sca14*) and *Pst*I and *Rsa*I (for *ompA*), were subsequently assessed *in vitro*. Digestion of the 407-bp, *htrA* amplicons was performed using the enzymes *Msp*I and *Rsa*I, as reported by (Santolin et al. (2013). Digestions were performed by combining 5 μL of amplicon, 1.2 μL of the appropriate reaction buffer, 1.2 units of restriction endonuclease, 1.2 μL of bovine serum albumin (0.1 mg/mL) and molecular biology grade water to a final volume of 12 μL. Incubation was at 37 °C for 3 h, followed by electrophoresis in agarose gels (2.5%) at a constant voltage of 5 V/cm for 90 min. Gels were stained with ethidium bromide, observed and digitally photographed under ultraviolet illumination. Banding patterns were compared between samples and via comparison to a DNA molecular weight marker (GeneRuler, 50 bp DNA Ladder). The resulting images were analyzed using the free software Gel-Analyzer (Skosyrev et al., 2013), available at http://www.gelanalyzer.com, to accurately determine the size of individual restriction fragments.

3. Results

The primer sets designed for the amplification of the four *sca* gene targets were considered specific for *R. bellii* based on *in silico* analysis and because they did not produce amplicons with the *Rickettsia*-negative tick extracts (n = 52), or with DNA purified from any of the other species of *Rickettsia* tested. Limiting dilution analysis of the control *R. bellii* (strain Mogi) DNA using the *htrA* assay, demonstrated the presence of amplifiable rickettsial DNA in samples diluted to a maximum of 10^{-5}. The relative sensitivity of the four *sca* gene assays was determined using the same diluted DNA samples and demonstrated that the *sca9* and *sca14* assays were of equivalent sensitivity to the *htrA* assay. In contrast, the 10^{-4} sample was the last dilution to be amplified by the *sca3* and *sca8* assays and therefore those primer sets were not examined in subsequent experiments employing field samples. The *sca9* and *sca14* assays produced amplicons with the predicted sizes from the seven positive control *A. rotundatum* ticks naturally infected with *R. bellii*, indicating the suitability of both assays for the examination of field samples. However, it was observed that all samples produced bands of lower intensity in the *sca14* assay.

The sensitivity of the novel *ompA* assay was determined to be equivalent to that of the *htrA* assay for each of the positive control DNAs examined. Specificity of the new primers was confirmed by the observation that they did not generate amplicon from the control *R. felis* and *R. bellii* DNAs or from the seven *R. bellii* infected *A. rotundatum* samples. The amplicon generated from the *R. monteiroi* control DNA

Table 2
PCR-RFLP fragment sizes predicted following *in silico* endonuclease digestion of a 357-bp amplicon of the gene *ompA*, using the enzymes *Pst*I and *Rsa*I.

Rickettsia species	Banding pattern (bp)	
	*Pst*I	*Rsa*I
Rickettsia amblyommatis	295, 62	156, 108, 93
'*Candidatus* Rickettsia andeanae'	295, 62	264, 93
'*Candidatus* Rickettsia colombianensi'	222, 135	357
R. monteiroi	264,72, 24	357
Rickettsia parkeri	135, 81, 78, 66	264, 93
Rickettsia rhipicephali	221, 139	159, 108, 93
Rickettsia rickettsii	140, 139, 81	159, 108, 93

was sequenced and showed 85% identity (272/320 nucleotides) to the sequences KT899079 and KU361217, deposited in the GenBank as partial sequences of '*Candidatus* Rickettsia tarasevichiae' outer membrane protein A (*ompA*) gene, amplified by PCR from *Ixodes persulcatus* ticks in Russia and Mongolia respectively. In addition, the novel sequence showed 81% identity (263/323 nucleotides) to the outer membrane protein A (*ompA*) gene, contained in the complete genome of *Rickettsia canadensis* strain McKiel, (CP000409.1). The novel *ompA* sequence was deposited in the GenBank with the accession number MK166031.

In silico restriction digest analysis, of predicted *ompA* amplicons, indicated that the enzymes *Pst*I and *Rsa*I would permit the differential identification of the SFG *Rickettsia* detected to date in Brazil (Table 2). *in vitro* digestion of amplicons of *R. amblyommatis*, *R. monteiroi*, *R. parkeri*, *R. rhipicephali*, *R. rickettsii* and '*Candidatus* Rickettsia andeanae' confirmed the discriminatory capacity of the dual enzyme PCR-RFLP approach (Fig. 1). DNA from '*Candidatus* Rickettsia colombianensi'was not available for use *in vitro* analysis.

The 150 DNA samples (Supplementary data Table S1), were examined using the *sac9* and *sca14* assays. A total of 12 samples (8%), comprising nine (9) *A. longirostre* ticks and a single *A. calcaratum* tick infected with *R. amblyommatis*, together with two (2) *Amblyomma* sp. haplotype Nazaré ticks infected with *R. parkeri* (strain ApPR), generated amplicons of the expected size in both assays (Fig. 2a). Yet, as noted previously with the *A. rotundatum* samples, the bands generated in the *sca14* assay were less intense than in the *sca9* assay.

The presence of SFG rickettsial DNA in all 12 samples was confirmed using the novel *ompA* assay (Fig. 3a). The *sca9* and *sca14* amplicons were sequenced and corresponded to the predicted fragments of their respective gene targets, confirming the presence of rickettsial co-infections in all 12 samples. The presence of a single nucleotide polymorphism (SNP), resulting in a synonymous mutation at nucleotide position 1193 of the full-length *sca9* gene (Accession number AY973497), was detected in the specimen of *A. calcaratum* collected in the state of Rio de Janeiro in 2015. The novel sequence was deposited in the GenBank with the accession number MK253742. Digestion of the *sca9* amplicons with the enzyme *Alu*I, produced a banding pattern of 396, 185, 82, 46 and 18 bp, identical to that recorded for amplicon produced from the *R. bellii* (strain Mogi), positive control DNA (Fig. 2b). Digestion of the *ompA* amplicons confirmed that the nine *A. longirostre* and the single *A. calcaratum* were infected with *R. amblyommatis* and that the two *Amblyomma* sp. haplotype Nazaré ticks were infected with *R. parkeri* (Fig. 3b).

4. Discussion

Brazilian wild birds are hosts to large populations of immature stages of *Amblyomma* ticks, with several records of infection with *R. bellii* and a variety of SFG *Rickettsia* in individual ticks collected from birds in diverse biomes (Ogrzewalska and Pinter, 2016; Luz et al., 2017; Ogrzewalska et al., 2010, 2011). A small number of studies have reported birds parasitized by multiple species of ticks, that in turn were found to be infected with distinct species of *Rickettsia* (Luz et al., 2017; Zeringóta et al., 2017). However, to our knowledge, there are no published records of co-infection of bird ticks with two or more species of *Rickettsia*. The data presented herein demonstrated for the first time that *A. longirostre* and *A. calcaratum* ticks can be naturally co-infected with *R. amblyommatis* and *R. bellii* and that *Amblyomma* sp. haplotype Nazaré ticks can simultaneously harbor the *Rickettsia parkeri*-like bacterium strain ApPR (Pacheco et al., 2012) and *R. bellii*. Indeed, our data represent the first records of *R. bellii* in *A. calcaratum* and in haplotype Nazaré ticks. As such, the number of tick species known to be infected with this agent in Brazil is now sixteen.

The experimental design commonly used to screen ticks for *Rickettsia* has not changed significantly since the first studies that reported molecular methods for that purpose (Bouyer et al., 2001; Gage et al., 1994). The general strategy involves a first round of PCR amplification of extracted DNA from all samples, using primers targeting genus-specific genes, normally *gltA* and/or *htrA*. Ticks positive for the first round PCRs are subsequently screened for the *ompA* gene, considered specific for members of the SFG (Fournier et al., 2003), coupled to nucleotide sequencing of all *ompA* amplicons to obtain species-level identifications. In studies involving large numbers of rickettsia positive samples, sequencing of amplicons from the first round of PCRs may be limited to the samples that were *ompA* negative, with such ticks frequently found to be infected with *R. bellii* (Luz et al., 2017; McIntosh et al., 2015). Following analysis of *ompA* sequences, it is common practice to sequence only a representative subset of the first round PCR products, to avoid redundancy and to reduce costs (Luz et al., 2017; Zeringóta et al., 2017). Using this strategy, the detection of co-infections with *R. bellii* becomes essentially a game of chance wherein the only criteria employed is the presence of double peaks in sequencing electropherograms that were derived from a small portion of the SFG infected ticks.

Previous attempts to sequence *gltA* fragments amplified from two of

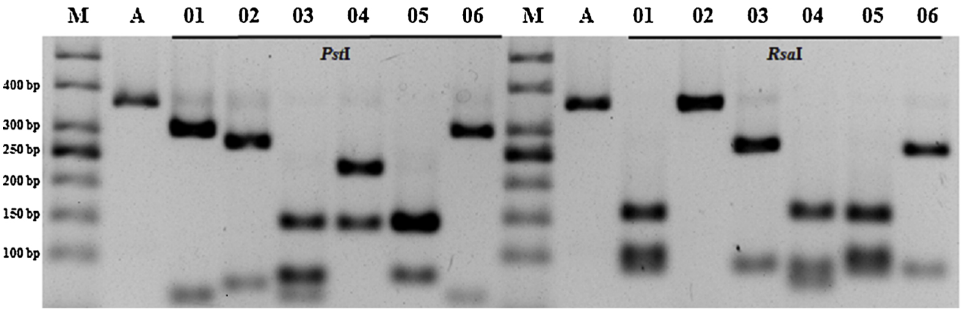

Fig. 1. Banding profiles generated following digestion of *ompA* amplicons, derived from *Rickettsia* species, using the enzymes *Pst*I and *Rsa*I. Lanes: A – undigested amplicon; 01 – *Rickettsia amblyommatis*; 02 – *Rickettsia monteiroi*; 03 – *Rickettsia parkeri*; 04 – *Rickettsia rhipicephali*; 05 – *Rickettsia rickettsii*; 06 – '*Candidatus* Rickettsia andeanae'. M = 50 bp DNA ladder. Molecular weights are indicated in base pairs (bp) on the left-hand side of the gel. Eletrophoresis was performed in a 2. 5% gel prepared in Tris-Boric acid-EDTA buffer (TBE 0.5X). Fragment sizes were determined by comparison to the bands contained in the molecular weight ladder.

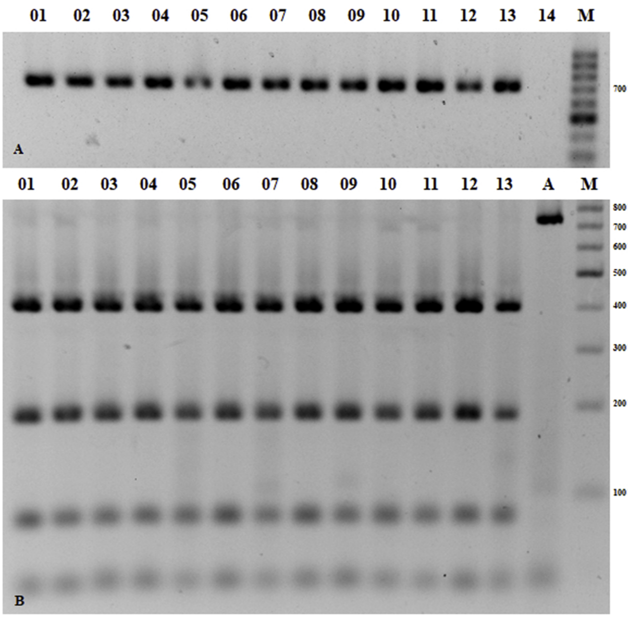

Fig. 2. A – PCR amplification of *sca9* targets in DNA extracted from nine *A. longirostre*, a single *A. calcaratum* and two *Amblyomma* sp. haplotype Nazaré ticks collected from wild birds in Brazil. Lanes 01 - 3133.19; 02 - 3122.16; 03 - 3160.1; 04 - 3130.8; 05 - L36; 06 - V22A; 07 - V5D; 08 - V7C; 09 - V7D; 10 - 3130.18; 11 - 35NAZ; 12 - 13bNAZ; 13 - *Rickettsia bellii* control DNA 14- H_2O negative control. M = 100 bp ladder. Electrophoresis was performed in a 1.5% agarose gel prepared in Tris-acetic acid-EDTA buffer (TAE 0.5X). Molecular weights are indicated in base pairs (bp) on the right-hand side of the gel; **B** - Analysis of restriction digest patterns, generated for the *sca9* amplicons shown in Fig. 2a, using the enzyme *Alu*I. A = undigested amplicon; Lanes 01-13 = *sca9* amplicons as identified in Fig. 2a. M = 100 bp DNA ladder. Molecular weights are indicated in base pairs (bp) on the right-hand side of the gel. Electrophoresis was performed in a 2. 5% gel prepared in Tris-Boric acid-EDTA buffer (TBE 0.5X). Fragment sizes were determined by comparison to the bands contained in the molecular weight ladder.

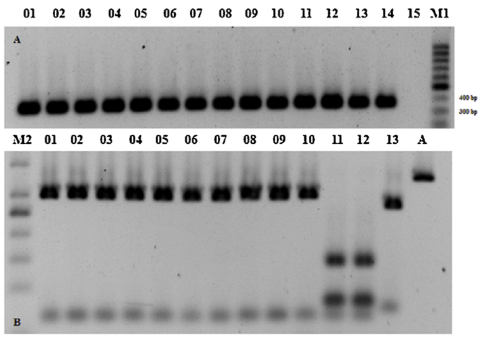

Fig. 3. A – PCR amplification of *ompA* targets in DNA extracted from nine *A. longirostre*, a single *A. calcaratum* and two *Amblyomma* sp. haplotype Nazaré ticks collected from wild birds in Brazil. Lanes 01 - 3133.19; 02 - 3122.16; 03 - 3160.1; 04 - 3130.8; 05 - L36; 06 - V22A; 07 - V5D; 08 - V7C; 09 - V7D; 10 - 3130.18; 11 - 35NAZ; 12 - 13b NAZ; 13 & 14 - *Rickettsia monteiroi* positive control DNA; 15- H_2O negative control. M1 = 100 bp ladder. Electrophoresis was performed in a 1.5% agarose gel prepared in Tris-acetic acid-EDTA buffer (TAE 0.5X). Molecular weights are indicated in base pairs (bp) on the right-hand side of the gel; **B** – Analysis of restriction digest patterns, generated for the *ompA* amplicons shown in Fig. 3a, using the enzyme *Pst*I. A = undigested amplicon; Lanes 01-13 = *ompA* amplicons as identified in Fig. 2a. M2 = 50 bp DNA ladder. Molecular weights are indicated in base pairs (bp) on the right-hand side of the gel. Electrophoresis was performed in a 2. 5% gel prepared in Tris-Boric acid-EDTA buffer (TBE 0.5X). Fragment sizes were determined by comparison to the bands contained in the molecular weight ladder.

the specimens of *A. longirostre* included in this study (collected in Minas Gerais in 2015 Zeringóta et al. (2017) and confirmed as infected with *R. amblyommatis* based on sequencing of fragments of *ompA* and *ompB*) had been unsuccessful, resulting in poor quality electropherograms which demonstrated double peaks at numerous locations throughout the sequence. The significance of that finding was not realized at the time of the original study. However, a careful re-analysis of the sequences indicated that both contained two distinct *gltA* amplicons, one corresponding to *R. amblyommatis* and the other to *R. bellii.* Indeed, it was the identification of those two co-infected samples which prompted the development of the methods described in the current work.

As in our earlier study (Zeringóta et al., 2017), such data may be overlooked or simply considered to represent poor-quality sequencing reactions. Through the removal of the factor of chance, the detection of co-infections with *R. bellii* will be enhanced and as a result, our comprehension of the role of *R. bellii* as a component of the bio-ecology of tick-borne rickettsial infections, including its potential role in rickettsial exclusion, should be improved.

As reported herein, the approach of using *sca9* and/or *sca14* as targets for first round PCR, in combination with the assay for *htrA*, provided a rapid method for the direct detection of *R. bellii* infections in *Amblyomma* ticks collected from wild birds, while also permitting the detection of DNA originating from the other members of the genus *Rickettsia* known to circulate in Brazil. Corroboration of the presence of *R. bellii* was rapidly (within 24 h) achieved by digestion of the *sca9* amplicons with the enzyme *Alu*I, or of the *Sca14* amplicons with *Eco*RV. Confirmation of the presence of DNA originating from an additional species of *Rickettsia* could be determined by digestion of the *htrA* amplicon as described by Santolin et al. (2013). To determine if the ticks were infected with an SFG *Rickettsia* and if so with which species, the novel *ompA* PCR was combined with RFLP analysis of amplicons using *Pst*I and *Rsa*I. The development of the novel *ompA* assay included in this study was considered necessary because an evaluation, using serially diluted SFG control DNAs, of the *ompA* assay routinely used by our group (employing the primers Rr190.70p and Rr190.602n), showed it to be 5 to 10-fold less sensitive than the *htrA* assay. Interestingly, the new *ompA* assay provided two unforeseen results; firstly, it permitted the differential identification by PCR-RFLP using the enzymes *Pst*I and *Rsa*I of the SFG agents detected in this study as well as of *R. rickettsii*. Secondly, it generated an amplicon for *R. monteiroi,* a rickettsial agent isolated from *A. incisum* ticks in Brazil that was placed in the canadensis group based on phylogenetic analysis of multiple gene sequences (Pacheco et al., 2011). Sequencing of the *ompA* amplicon supported the findings reported for the other genes.

It should be noted, that the inability of the *ompA* PCR to detect *R. felis* or *R. asembonensis* represented a potential shortcoming in the detection strategy, albeit one that can be countered by digestion of the 407-bp *htrA* amplicon with the enzyme *Msp*I, resulting in the differential banding profile of 200, 157 and 50 bp as reported by Santolin et al. (2013). It is important to note, that the use of PCR-RFLP for post-amplification identification of *Rickettsia* was commonplace in the past (Eremeeva et al., 1994; Gage et al., 1994; Peniche-Lara et al., 2013) but has largely been replaced, during the last decade, by sequencing of amplicons. Nonetheless, even with the on-going reduction in the costs associated with sequencing, it is clear that the use of PCR-RFLP should be recognized as a convenient means of cutting both the costs and the time taken to establish a robust identification of tick-borne *Rickettsia* in Brazil, particularly in situations where sequencing would involve outsourcing to third party service providers (Santolin et al., 2013).

The rickettsial exclusion theory was established via simple, yet refined, experiments and is widely accepted by the majority of rickettsiologists. Nevertheless, there are some gaps in the theory, which were elegantly examined in the evenhanded review of the evidence by Telford (2009) and in the more recent data presented by Levin et al. (2018). Moreover, the application of next-generation sequencing

(NGS), technologies to the examination of tick microbiomes has indicated that the exclusion effect may not be limited to rickettsia-rickettsia interactions but rather it may also involve interactions between other components of the microbiota including facultative intracellular *Francisella*-like endosymbionts (Gurfield et al., 2017). It has even been suggested, also based on NGS analysis of tick microbiomes, that co-infection of ticks with *Rickettsia* and other pathogenic microbes (*Anaplasma*, *Babesia*, *Bartonella* and *Borrelia*), and/or endosymbionts (*Candidatus* Midichloria, *Spiroplasma*, and *Wolbachia*), may be the rule rather than the exception (Cerutti et al., 2018; Moutailler et al., 2016). Deciphering the biological significance of such interactions is a daunting task. However, it has recently been reported that the presence of "*Candidatus* Midichloria mitochondrii" in conjunction with selenoproteins, was essential for the growth of *R. parkeri* in *A. maculatum*, the gulf coast tick (Budachetri et al., 2018), demonstrating that improvements in our comprehension of microbe-microbe interactions in arthropod vectors of human and animal disease, may provide the basis for novel control strategies via microbiome and/or metabolome manipulation (Cabezas-Cruz et al., 2018; de la Fuente et al., 2017).

Unfortunately, the destructive nature of the DNA extraction method used herein, prevented an assessment of the tissue distribution of the co-infecting bacteria. Thus, it was impossible to reach any conclusions regarding the possible rickettsial exclusion capabilities of *R. bellii*. Although both technically and logistically demanding, future studies could be designed wherein tick samples would be collected and processed to produce histological sections for evaluation by immunofluorescence (Hirunkanokpun et al., 2011) and/or *in situ* fluorescent hybridization (Bagheri et al., 2017), to determine the tissue localization of the different rickettsial agents within individual ticks, as was reported for fleas infected by *R. felis* (Hirunkanokpun et al., 2011) and for *Ixodes pacificus* ticks infected with a variety of rickettsial agents (Bagheri et al., 2017). Subsequent evaluation of DNA extracted from sectioned material, using the *sca9/ompA* PCR-RFLP approach would provide confirmation of co-infection together with species-level identification of the *Rickettsia* species contained therein.

Rickettsia bellii is the earliest divergent known species of Rickettsia and demonstrates, by far, the widest host range in the American continents having been reported in association with 25 different tick species (Costa et al., 2017). However, our general understanding of the bacterium (including its potential to influence the biology of the host or its interactions with other components of the microbiome), is far from complete. The findings of the current work indicate that the distribution of *R. bellii* in Brazilian tick populations, particularly as a co-infecting species, is most likely underestimated. It is envisaged that the adoption of the methods reported herein will enhance our capacity to accurately detect the occurrence of co-infections and may serve to improve our comprehension of the role of *R. bellii* in the bio-ecology of tick-associated *Rickettsia*, including its potential participation in rickettsial exclusion.

Declaration of Competing Interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2019.101266.

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