DETECTION AND IDENTIFICATION OF Rickettsia AGENTS IN TICKS COLLECTED FROM WILD BIRDS IN BRAZIL BY POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP) ANALYSIS*

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ABSTRACT. Santolin I.D.A.C., Famadas K.M. & McIntosh D. Detection and identification of Rickettsia agents in ticks collected from wild birds in Brazil by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. [Detecção e identificação de espécies de Rickettsia em carrapatos coletados de aves silvestres no Brasil pela PCR-RFLP.] Revista Brasileira de Medicina Veterinária, 35(Supl.2):68-73, 2013. Programa de Pós-Graduação em Ciências Veterinárias, Instituto de Veterinária, Anexo 1, Universidade Federal Rural do Rio de Janeiro, Campus Seropédica, BR 465 Km 7, Seropédica, RJ 23897-970, Brasil. E-mail: isis@santolin.com.br

The detection and identification of Rickettsia bacteria in tick populations have become easier and more precise during the last 25 years as result of the development of robust molecular biology based methods. Currently, seven species of Rickettsia have been described in Brazilian ticks, using PCR for detection and amplicon sequencing for identification. In silico analysis of sequences encoding the gltA, htrA and ompB genes of those species was performed and revealed the basis for a novel PCR-RFLP method that allows their differential identification. The method was evaluated using larvae and nymphs of Amblyomma longirostre, A. ovale and A. varum collected from birds in the Tinguá Biological Reserve, in Rio de Janeiro State. The species “Candidatus Rickettsia amblyommii” was identified in 100% of the A. longirostre examined, while the other two tick species were PCR negative. The basic method employs the restriction endonucleases Msp1 and RsaI and can be performed in the course of a single working day. It offers a convenient and cost effective means to perform large scale analysis of tick populations, and should be of benefit to researchers who lack the financial or technical resources necessary for sequence based identification.

KEY WORDS. Rickettsia, detection, PCR, ticks.

RESUMO. Rickettsioses transmitidas por carrapatos são doenças zoonóticas graves e potencialmente ameaçadoras à vida. A detecção e identificação de bactérias do gênero Rickettsia em populações de carrapatos tornaram-se mais fáceis e precisas nos últimos 25 anos, devido ao desenvolvimento de métodos baseados em biologia molecular. Atualmente, sete espécies de Rickettsia foram descritas nos carrapatos brasileiros, utilizando PCR para a detecção e sequenciamento de “amplicons” para a identificação. A análise in silico de sequências que codificam os genes gltA, htrA e ompB dessas espécies foi realizada e revelou ser a base para um novo método de PCR-RFLP, que permite a identificação diferencial das sete espécies brasileiras. O método foi avaliado utilizando larvas e ninhas de


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Amblyomma longirostre, Amblyomma ovale e Amblyomma varium coletadas em aves na Reserva Biológica do Tinguá, no estado do Rio de Janeiro. A espécie “Candidatus Rickettsia amblyommii” foi identificada em 100% dos A. longirostre examinados, enquanto que as outras duas espécies de carrapatos foram PCR negativos. O método básico emprega as endonucleases de restrição MspI e Rsal e pode ser realizada no decorrer de um único dia de trabalho. Dispõe de um conveniente e rentável meio para executar a análise em grande escala de populações de carrapatos, e deve ser um benefício para os pesquisadores que não dispõem de recursos financeiros ou técnicos necessários para a identificação baseado no sequenciamento.

PALAVRAS-CHAVE. Rickettsia, detecção, PCR, carrapatos.

INTRODUCTION

Tick-borne rickettsioses are zoonotic infections, with a global distribution, caused by intracellular bacteria belonging to the spotted fever group (SFG) of the genus Rickettsia (Parola et al. 2013). However, due to shared symptoms with other bacterial and viral infections, it is thought that many human rickettsial infections are misdiagnosed (Abbad et al. 2011). Rickettsial infections of ticks can be determined by immunological, cell culture or molecular biology based methods including PCR, PCR-RFLP, isothermal amplification techniques and increasingly by nucleotide sequencing (Parola et al. 2013). It is of interest to note, that prior to 2004 the only tick-associated rickettsia species documented in Brazil was R. rickettsii, the etiological agent of Brazilian spotted fever (BSF). However, during the last decade an additional 6 species (“Candidatus Rickettsia amblyommii”, R. bellii, R. monteiroi, R. parkeri, R. rhipicephali and Rickettsia sp. Pampulha strain), have been detected in a variety of tick species, parasitizing a range of hosts including wild animals and birds (Labruna et al. 2011, Parola et al. 2013). In this context, the species “Candidatus Rickettsia amblyommii” has emerged as the Rickettsia most frequently isolated from bird ticks in Brazil and has been identified in Amblyomma cajennense, A. coelebs, A. geayi, A. auricularium and Amblyomma longirostre (Ogrzewalska et al. 2010, Labruna et al. 2004a, Labruna et al. 2011, Parola et al. 2013, Saraiva et al. 2013). The only other species of SFG Rickettsia detected in bird ticks in Brazil to date has been R. parkeri (Ogrzewalska et al. 2011a, Ogrzewalska et al. 2013, Pacheco et al. 2012).

The pathogenic potential for humans of the more recently isolated species is unclear. Yet, it is pertinent to note that data from serological surveillance of dogs in the Patanal region of Brazil, provided evidence for infection by R. amblyommii most likely via the tick A. cajennense (Melo et al. 2011). Moreover, a subsequent study of birds and other wild animals in the Atlantic Forest in São Paulo state, Brazil (Ogrzewalska et al. 2012), also recorded high levels of sero-conversion for R. amblyommii in small mammals and detected R. amblyommii by PCR in 41.7% of the A. longirostre ticks collected from birds, demonstrating that this Rickettsia spp. is widely distributed and is circulating within wild and domestic animal populations in Brazil.

Significant advances in our understanding of the biology, ecology and epidemiology of tick-borne rickettsioses in Brazil have been made in the last decade (Parola et al. 2013). Nevertheless, continued progress in this field will depend upon the performance of large scale surveys of tick populations using molecular detection and identification methods. The costs and high level of technical ability required to competently undertake sequencing based analysis, serve to make the use of this approach impractical for many laboratories. In an attempt to address this constraint, the current study employed bioinformatical methods to develop a robust and cost effective molecular detection protocol, based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis, which can be used as an alternative to sequencing. In addition, we report the application of this method to the detection and identification of Rickettsia in A. longirostre ticks collected from birds in the State of Rio de Janeiro, Brazil.

MATERIALS AND METHODS

DNA derived from cell cultures or tick extracts previously determined by PCR and sequence analysis to be positive for ‘Candidatus Rickettsia amblyommii’ (strain ARANHA), R. bellii, R. felis or R. parkeri (strain NOD), were used as positive controls for all PCR and restriction digests. Amplification of a 401 base pairs (bp), fragment of the citrate synthase gene gltA, which is conserved in all Rickettsia species reported to date in Brazil, was performed using the primers (forward) CS-78 and (reverse) CS-323 (Labruna et al. 2004b). The primers 17KDF (5’GGAAACGCGGGGATTAATA’3’) and 17KDR (5’ACTTTGCGCATGTCGTCAGG 3’), were employed to amplify a 407 bp fragment of the genus specific gene htrA and the primers 120-M59F and 120-807, were used to amplify an 856-bp fragment of the rickettsial 135-kDa outer membrane protein gene (ompB)
Despite the significant progress of recent years, there are still numerous gaps in our understanding of the biology of tick-borne *Rickettsia* in Brazil (Szabó et al. 2013). In order to fill those gaps, there is a pressing need to perform extensive surveillance of Brazilian tick populations for the presence of *Ri-
agents (Ogrzewalska et al. 2012, Parola et al. 2013). On the one hand this could be simply and readily achieved, at least in terms of total detection rates, via PCR based screening with genus specific primers as employed in the current study. On the other hand, accurate identification at the species or sub-species levels (as required for detailed epidemiological investigations, identification of outbreak strains, or phylogenetic studies), requires post amplification analysis in the form of sequencing of PCR products (Parola et al. 2013). Unfortunately, in spite of continual reductions in the cost of sequencing methodologies, the routine application of sequencing as a technique for bacterial identification is still not financially viable or practical for many laboratories, owing to the technical intricacies and training required to perform proficient analysis of large quantities of sequencing data. In light of those restraints, the application of alternative and less expensive methods for analysis of PCR products should be advocated as a means of performing preliminary molecular analysis of isolates and in order to select representative groups of tick derived amplicons for sequencing. One such alternative method is PCR-RFLP, which relies upon restriction endonuclease based digestion of PCR amplicons to generate a series of differently sized fragments, which occur as a result of mutations in restriction site sequences, the acquisition or deletion of recognition sites, or insertions and deletions in the sequences between recognition sites (Rameshkumar et al. 2012). The digested DNA is separated electrophoretically in gels (agarose or polyacrylamide) and the image of the gel is recorded digitally for posterior analysis of banding patterns. (Rameshkumar et al. 2012). The acquisition or deletion of recognition sites, or results of mutations in restriction site sequences, are capable of providing differential identification of the seven species of \textit{Rickettsia} reported from Brazilian ticks, as well as \textit{R. massiliae}, which has been reported in ticks from Argentina (Parola et al. 2013) and \textit{R. felis}. The species \textit{R. felis} is generally considered to be carried by fleas, but a number of alternative arthropod vectors for this bacterium, including ticks, have recently been reported in a variety of geographical locations (Abdad et al. 2011). As such, we considered it both prudent and justified to include this species in our analysis.

As shown in Table 1, the majority of the \textit{Rickettsia} species could be easily differentiated based upon comparison of the banding patterns generated by \textit{MspI} and \textit{RsaI} digestion of the \textit{ompB} amplicon. However, sequences corresponding to the 856 bp amplicon were not available for \textit{R. monteiroi} or for \textit{Rickettsia} species Pampulha strain. In addition, the primers used in the current study do not amplify the \textit{ompB} gene of \textit{R. bellii}. Nevertheless, differential identification based on unique banding patterns (Table. 1), could be achieved using \textit{MspI} and \textit{RsaI} to digest the \textit{htrA} amplicon of \textit{Rickettsia} species Pampulha strain, \textit{MspI} and \textit{SacI} to cut the \textit{htrA} amplicon of \textit{R. monteiroi} and \textit{HindIII} to cleave the \textit{htrA} amplicon of \textit{R. bellii}.

To assess the utility of this method with field

<table>
<thead>
<tr>
<th>\textbf{Rickettsia Species}</th>
<th>\textbf{Fragment sizes in base pairs generated from 407 bp amplicon}</th>
<th>\textbf{Fragment sizes in base pairs generated from 856-bp amplicon of \textit{ompB}}</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Candidatus \textit{Rickettsia amblyomii}&quot;</td>
<td>250, 157, 298, 82, 27</td>
<td>505, 222, 129, 599, 174, 83</td>
</tr>
<tr>
<td>\textit{R. bellii}</td>
<td>305, 102, 149, 137, 54, 33, 34</td>
<td>NA, NA</td>
</tr>
<tr>
<td>\textit{R. felis}</td>
<td>200, 157, 50, 298, 82, 27</td>
<td>363, 309, 87, 63, 40, 599, 174, 89</td>
</tr>
<tr>
<td>\textit{R. massiliae}</td>
<td>250, 102, 95, 298, 82, 27</td>
<td>505, 222, 129, 559, 174, 83, 40</td>
</tr>
<tr>
<td>\textit{R. monteiroi}</td>
<td>407, 195, 97, 82, 33</td>
<td>NA, NA</td>
</tr>
<tr>
<td>\textit{R. parkeri}</td>
<td>250, 157, 298, 82, 27</td>
<td>505, 222, 129, 391, 169, 144, 83, 39, 30</td>
</tr>
<tr>
<td>\textit{R. rhipicephali}</td>
<td>250, 102, 55, 298, 82, 27</td>
<td>636, 220, 559, 174, 83, 40</td>
</tr>
<tr>
<td>\textit{R. rickettsii}</td>
<td>250, 157, 298, 82, 27</td>
<td>353, 222, 152, 129, 430, 169, 144, 83, 30</td>
</tr>
</tbody>
</table>

\textit{Rickettsia} sp. Pampulha strain | 200, 157, 50, 207, 82, 69, 22, 27 | NA, NA |

NA= no sequence available. * Digestion of the 407 bp amplicon \textit{htrA} amplicon of \textit{R. monteiroi} with the restriction endonuclease \textit{SacI} generates a unique banding profile based on two bands with molecular masses of 216 & 191bp.
samples, ticks were collected from wild birds and examined for the presence of amplifiable rickettsial DNA. DNA was extracted from a total of 19 ticks represented by 17 *A. longirostre* (13 larvae and 4 nymphs), one larvae of *A. ovale*, and a single nymph of *A. varium* collected from specimens of *Dendrocinclia tordina*, *Sittasomus griseicapillus*, *Xiphorhynchus fuscus*, and *Xiphorhynchus guttatus*. All (17/17) of the *A. longirostre* generated amplicons of the predicted size when examined using each of the three PCR assays. In contrast, the other two tick species were recorded as universally negative for amplifiable rickettsial DNA. Subsequent digestion of the 17 *ompB* amplicons with *MspI* and *RsaI* revealed that in all cases the ticks were infected with “*Candidatus Rickettsia amblyommii*” (Figure 1). This finding was not unexpected given the predominance of this species in the majority of studies on birdtick-associated rickettsia conducted in Brazil (Ogrzewalska et al. 2008, Ogrzewalska et al. 2010, Ogrzewalska et al. 2011b, Ogrzewalska et al. 2012 Labruna et al. 2011). It is of interest that, based upon sequencing data, two genetic variants of “*Candidatus Rickettsia amblyommii*” denominated strains ARANHA and AL have been described infecting immature stages of *A. longirostre* in Brazil (Parola et al. 2013). Differential identification of the three “species” could not be attained using digestion of the *ompB* amplicon. However, it was observed that all of the *htrA* sequence deposited in the GenBank as being derived from “*Candidatus Rickettsia amblyommii*” contained a single site for the enzyme HindIII which upon digestion would yield two fragments of 203 and 204 base pairs in size. This restriction site was found to be absent from the sequences deposited for strains ARA-NHA (GenBank accession number AY360215), and AL (GenBank accession number EU274655).

As such, PCR-RFLP using this sequence and the enzyme HindIII, provides a possible means for differentiation between the classical strain and the Brazilian variants. The *htrA* amplicons from our tick samples were not cut upon digestion with HindIII, indicating that they were either strain ARA-NHA or AL.

In conclusion, we report the development of a simple and robust PCR-RFLP protocol, which is based upon widely employed rickettsial gene targets and PCR assays and that allows the rapid (24 to 48 hour), identification of each of the tick-associated *Rickettsia* species which have been reported to date in Brazil. The method is cost effective and provides a technically uncomplicated alternative to sequence based identification. It is our hope that the availability of this protocol may serve to stimulate increased research on the study of this important group of emerging and re-emerging pathogens.

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Detection and identification of *Rickettsia* agents in ticks collected from wild birds in Brazil


