Ticks and Tick-borne Diseases 7 (2016) 1061–1066

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Molecular analysis reveals the diversity of *Hepatozoon* species naturally infecting domestic dogs in a northern region of Brazil

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ARTICLE INFO

Article history:
Received 1 April 2016
Received in revised form
12 September 2016
Accepted 18 September 2016
Available online 20 September 2016

Keywords:
Hepatozoon canis
Hepatozoon americanum
Canine hepatozoonosis
Nested PCR
Molecular epidemiology

ABSTRACT

This study aimed to optimize molecular methods for detecting DNA of *Hepatozoon* spp. as well as identify the phylogenetic relationships of *Hepatozoon* strains naturally infecting domestic dogs in Belém, Pará, northern Brazil. Blood samples were collected from 138 dogs, and screened for *Hepatozoon* spp. using a new nested PCR assay. Positive samples were subjected to genetic characterization based on amplification and sequencing of approximately 670 bp of the *Hepatozoon* spp. 18S rRNA. Of the positive dogs, four shared the haplotype Belém 01, one dog presented the haplotype Belém 02 and two dogs shared the haplotype Belém 03. A Bayesian inference indicates that haplotypes Belém 01 and Belém 02 are phylogenetically related to *H. canis*, while Belém 03 is related to *H. americanum*. Overall, based on the first molecular evidence of *H. americanum* in Brazilian domestic dogs, the proposed protocol may improve the epidemiological investigation of canine hepatozoonosis.

1. Introduction

Hepatozoonosis is a tick-borne disease of wild and domestic carnivores caused by the *Hepatozoon* spp. (Apicomplexa). These protozoa are intracellular parasites that infect leukocytes of the host animals. Since canine hepatozoonosis is the most well described of the *Hepatozoon* infections, *Hepatozoon canis* and *Hepatozoon americanum* species are recognized as its etiologic agents (Smith, 1996; Vincent-Johnson et al., 1997; Baneth, 2011).

H. canis is known to be transmitted by ticks *Amblyomma ovale* (Forlano et al., 2005, 2007; Rubini et al., 2009), *Haemaphysalis longicornis*, *Haemaphysalis flava* (Murata et al., 1995), and mainly by the brown dog tick *Rhipicephalus sanguineus* sensu lato (Baneth et al., 1998), which is found in tropical and subtropical regions around the world, including the United States and Brazil (Dantas-Torres, 2010). In Brazil, the participation of *R. sanguineus* sensu lato ticks in the transmission of *H. canis* to dogs needs comprehensive investigation. Many studies indicate the existence of a "*R. sanguineus* group" (Szabó et al., 2005; Dantas-Torres and Otranto, 2015; Nava et al., 2015), which suggests that the vector competence of this tick species for *H. canis* might differ among geographical regions (Dantas-Torres and Otranto, 2015) and may explain why in Brazil, until now, *R. sanguineus* sensu lato was not found infected with *H. canis,* neither naturally nor experimentally (Rubini et al., 2009; Demoner et al., 2013). Recently, Melo et al. (2016) detected *H. canis* DNA in *Amblyomma cajennense* sensu lato ticks, but they did not discard the possibility that the DNA origin was the ingested blood.

So far, the only known vector of *H. americanum* has been the Gulf Coast tick *Amblyomma maculatum* (Mathew et al., 1998; Panciera et al., 1999; Ewing et al., 2002; Vincent-Johnson, 2003), which is originally a Nearctic species, but has been reported in Central and South American countries (Guglielmone et al., 2003; Voltzit, 2007; Teel et al., 2010).

In general, dogs infected with *H. canis* are asymptomatic or present a subclinical infection named Old World Canine Hepato-

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http://dx.doi.org/10.1016/j.ttbdis.2016.09.008
1877-959X/

zoonosis (OWCH); however, high parasite burdens can result in a syndrome characterized by lethargy, anemia and cachexia (Rubini et al., 2005), which is most characteristic of American Canine Hepatozoonosis (ACH), the syndrome caused by *H. americanum* infection. While OWCH shows clinical signs ranging from mild to moderate (Baneth, 2011), ACH is characterized by severe symptoms that lead to debilitation and death. It is worth noting that because the *H. canis* infection is often associated with other infectious agents transmitted by ticks, the clinical signs and hematological abnormalities presented by dogs with OWCH cannot be attributed solely to *H. canis* infection (Ewing et al., 2000; Sasanelli et al., 2009; O'Dwyer, 2011).

The diagnosis of canine hepatozoonosis includes direct detection as well as indirect detection. Direct detection is usually based on the presence of gamonts in routine blood smear preparations (Baneth et al., 2003; Baneth and Vincent-Johnson, 2005; O'Dwyer et al., 2006; Eiras et al., 2007; Mundim et al., 2008) and cysts (merogonic stages) found in the internal organs of the host (Craig et al., 1984; Baneth and Shkap 2003; Cardoso et al., 2014). Although blood smear analysis is an easy and inexpensive method, this method can result in false negative diagnoses, especially in cases where the parasitemia is very low or intermittent (Karagenc et al., 2006). In fact, dogs with *H. americanum* infection characteristically have a lower parasitemia than dogs with *H. canis* infection (Elias and Homans 1988; Baneth et al., 1996; Vincent-Johnson et al., 1997; Gondim et al., 1998).

Molecular analysis based on single round standard polymerase chain reaction (PCR) targeting partial 18S rRNA gene of the *Hepatozoon* spp. is an alternative and sensitive method to directly diagnose *Hepatozoon* infections (Inokuma et al., 2002; Criado-Fornelio et al., 2003; Otranto et al., 2011; Aydin et al., 2015). However, the sensitivity of this approach can be further enhanced by the application of other techniques such as nested polymerase chain reaction (nPCR), semi-nested polymerase chain reaction (snPCR) or real-time polymerase chain reaction (qPCR), which can be especially useful when parasitemia is low. A qPCR assay developed by Li et al. (2008) proved to be a valuable tool that can improve detection of *Hepatozoon* spp. DNA, although this method is not broadly available due to its high costs.

Indirect diagnoses, which are based on serological tests such as enzyme-linked immunosorbent assay (ELISA) or indirect fluorescent antibody test (IFAT) are more sensitive than microscopic analysis of peripheral blood (Shkap et al., 1994; Karagenc et al., 2006). According to O'Dwyer (2011), serological methods are valuable for epidemiological studies and for diagnosing infected dogs, however these techniques need an antigen source consisting of samples obtained from dogs with extremely high parasitemia, and thus they present limitations that may hamper the diagnosis.

Currently, canine hepatozoonosis has been not reported in northern Brazil. Thus, the present study was undertaken in order to optimize a molecular method to detect the DNA of *Hepatozoon* spp. and identify the phylogenetic diversity of *Hepatozoon* strains naturally infecting domestic dogs in Belém, Pará, northern Brazil. It is highly relevant due to the severity of the disease caused by coinfection of *Hepatozoon* spp. and other pathogens, which are known to occur in this region (see Rufino et al., 2013; Moraes et al., 2014).

2. Materials and methods

2.1. Samples and DNA extraction

A total of 138 samples were randomly selected from dogs that were treated at the Veterinary Hospital of the Universidade Federal Rural da Amazônia (HOVET-UFRA), Belém, Pará State between August and October 2011. Blood samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA). Total DNA of each sample was extracted from a 300 μL aliquot of the blood by using a standard phenol-chloroform procedure, as described by Sambrook et al. (1989). DNA quality was checked by electrophoresis on an agarose gel, and the DNA was then quantified using the Qubit 2.0 fluorometer (Thermo Fisher Scientific).

2.2. PCR amplification and DNA sequencing

Molecular diagnosis of *Hepatozoon* spp. was improved by using a new nested PCR assay based on the partial amplification of the 18S rRNA gene of *Hepatozoon* spp. We used HepF and HepR primers (Inokuma et al., 2002) in the first round of PCR followed by a second round of PCR with HepNF (5′GGTATGGTATTGGCTTACCG3′) and HepNR (5′CGAGCTTTTTAACTGCAACA3′) primers. HepNF and HepNR were designed by using the Primer3Plus software (Untergasser et al., 2007) based on nucleotide sequences of *Hepatozoon* spp. retrieved from the GenBank database (NCBI), whose accession numbers were AY150067 (Criado-Fornelio et al., 2003), JX466884 (Duscher et al., 2013), HQ829438 (unpublished), EU028344 (Metzger et al., 2008), AF176836 (Mathew et al., 2000), JN181157 (Barta et al., 2012) and EU041717 (Kubo et al., 2008).

The first round of amplification was carried out in 25 μL reactions with 10–20 ng of the DNA template, 1.5 mM $MgCl_2$, 0.075 mM each of deoxyribonucleotide triphosphates (dNTPs), 10 mM Tris-HCl, 50 mM KCl, 0.2 μM of each primer (HepF and HepR), and 1 U Taq DNA polymerase (Invitrogen). The amplification reaction consisted of 35 cycles of 30 s at 95 °C, 30 s at 57 °C, and 1 min at 72 °C, preceded by 5 min at 95 °C and followed by 10 min at 72 °C. The second round of amplification was also carried out in 25 μL reactions with 1 μL of the first-round PCR product, 1.5 mM of $MgCl_2$, 0.075 mM each of dNTPs, 10 mM Tris-HCl, 50 mM KCl, 0.2 μM of each primer (HepNF and HepNR), and 1 U Taq DNA polymerase (Invitrogen). The thermal and time conditions were as described above except for the cycle numbers that were 30. All the PCR products were visualized after electrophoresis in 1.5% agarose gel in Tris-acetate-EDTA (TAE) buffer, using GelRedTM Nucleic Acid stain (Biotium) and an ultraviolet transilluminator. A 100 bp molecular marker (Invitrogen DNA ladder) was used to estimate the size of each amplified fragment. The samples that produced fragment size of 309 bp for the second round PCR were considered to be positive and then subjected to analysis of taxonomic diversity.

In order to properly identify the *Hepatozoon* species we performed another nested PCR assay to amplify a larger fragment (~670 bp) of the 18S rRNA than was amplified in the molecular diagnosis protocol. In this second assay, the first round of amplification included universal apicomplexan primers: forward (5′AACCTGGTTGATCCTGCCAGTAGTCAT3′) and reverse (5′GAATGATCCTTCCGCAGGTTCACCTAC3′) (Cacciò et al., 2002), while the second round PCR included HepF and HepR primers. First round PCR was carried out in 25 μL reactions with 10–20 ng of the DNA template, 2.5 mM $MgCl_2$, 0.125 mM each of deoxyribonucleotide triphosphates (dNTPs), 10 mM Tris-HCl, 50 mM KCl, 0.15 μM of each primer, and 1 U Taq DNA polymerase (Invitrogen). The amplification reaction consisted of 40 cycles of 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C, preceded by 5 min at 95 °C and followed by 2 min at 72 °C. Second round PCR was carried out under the same conditions of the first round PCR used here to the molecular diagnosis, excepting the DNA samples were changed by 1 μL of the PCR product amplified with universal apicomplexan primers cited above.

For both analyses (i.e. molecular diagnosis and taxonomic diversity), DNA of *H. canis* was used as the positive control, while sterile bi-distilled water was used as the negative control.

Amplicons of the second round PCR (~670 bp) of the second nested PCR assay were excised from the agarose gel, and then

Table 1
Hepatozoon spp. 18S rRNA sequences used for phylogenetic analysis plus additional information retrieved from the GenBank database.

Strain	GenBank accession No.	Specie	Host	Reference
Spain	AY150067	*H. canis*	Fox	Criado-Fornelio et al. (2003)
Brazil	AY471615	*H. canis*	Fox	Criado-Fornelio et al. (2006)
Italy	KP644235	*H. canis*	Fox	Unpublished
Brazil	EF622096	*H. canis*	Capybara	Unpublished
Croatia	HM212626	*H. canis*	Fox	Dezdek et al. (2010)
Hungary	KJ572976	*H. canis*	Golden jackal	Farkas et al. (2014)
Croatia	FJ497022	*H. canis*	Dog	Vojta et al. (2009)
Italy	GU371447	*H. canis*	Fox	Gabrielli et al. (2010)
Brazil	KC127679	*Hepatozoon* sp.	Fox	Almeida et al. (2013)
USA	AF176836	*H. americanum*	Dog	Mathew et al. (2000)
USA	JX415176	*H. americanum*	Coyote	Unpublished

purified with a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). An aliquot of each purified product was ligated into a pGEM-T vector (Promega) overnight and then desalted before electroporation into *Escherichia coli* TOP10 (Thermo Fisher Scientific). The inserted DNA of white clones was obtained by PCR directly from the colonies using M13F/M13R primers. Nucleotide sequencing was performed in a ABI 3500 xL Genetic Analyzer (Thermo Fisher Scientific), according to the manufacturer's specifications. BioEdit software (Hall, 1999) was used to align forward and reverse sequences.

2.3. PCR sensitivity test

The positive control amplicon produced by the HepF and HepR primers was purified and cloned as described above for the samples that were sequenced. Cloned amplicon was subjected to first round PCR using the HepF and HepR primers and its product was quantified (45.9 ng/μL) by the Qubit 2.0 fluorometer (Thermo Fisher Scientific). An aliquot of 1 μL of this PCR product was diluted 9 times (from 10^{-1} to 10^{-9}) and sensitivity testing was performed by amplifying serial dilutions through HepNF and HepNR primers.

2.4. Data analysis

Simple PCR (using HepF and HepR only) and nested PCR (using HepNF and HepNR following the first round of amplification using HepF and HepR) sensitivities for the detection of *Hepatozoon* spp. 18S rRNA were compared by McNemar's test using BioEstat 5.3 software (Ayres et al., 2007).

In addition to sequences obtained here, the phylogenetic analysis was performed including thirteen *Hepatozoon* spp. 18S rRNA sequences retrieved from the GenBank database (Table 1), including two *Hepatozoon felis* 18S rRNA sequences which were used as the outgroup. Bayesian Inference (BI) was conducted based on Markov Chain Monte Carlo (MCMC) tree searches as implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). We conducted two parallel runs of four simultaneous MCMC searches for 5 million generations each, sampling one tree every 500 generations, and discarding the results of the first 1000 trees as burn-in. The remaining trees were used by MrBayes to estimate the posterior probability of each node in our phylogenetic reconstruction. Tracer v1.4.1 (Rambaut and Drummond, 2008) was used to check the stationarity of all parameters sampled by the chains. The p distances between pairwise sequences were performed using PAUP 4.0b10 (Swofford, 2002).

3. Results

All the DNA samples showed high purity and integrity. The positivity of used control was confirmed based in a comparison of the nucleotide sequence of the fragment amplified using the HepF and HepR primers with the sequence AY150067 (Criado-Fornelio et al., 2003).

A comparative analysis of the results of simple PCR and nested PCR revealed a significant statistical difference (p value = 0.0156). While no sample except the positive control was positive using the simple protocol, seven (~5.1%) proved to be positive using the nPCR approach. Moreover, amplification of serial dilutions revealed that HepNF and HepNR primers can detect approximately 0.459 fg/μL of *Hepatozoon* DNA (Supplementary Fig. 1).

The seven nucleotide sequences obtained resulted in 3 haplotypes; two of them had 664 nucleotides in length and were defined as Belém 01 (GenBank accession number KU729737) and Belém 02 (GenBank accession number KU729738), while the remaining haplotype with 668 nucleotides in length was defined as Belém 03 (GenBank accession number KU729739). In comparison to each other, haplotypes Belém 01 and Belém 02 differed by 5 substitutions, whose 4 were identified as transitions and 1 as transversion. The largest level of polymorphism was observed between haplotypes Belém 01 and 03, which presented 21 transitions, 13 transversions and 4 indels. Regarding the prevalence, four dogs shared haplotype Belém 01, one dog presented haplotype Belém 02 and two dogs shared haplotype Belém 03.

By using BLAST analysis, the three haplotypes we obtained are closely related to *Hepatozoon* 18S rRNA sequences. BI assumed a GTR + G model of nucleotide substitution with estimated base frequencies (A = 0.3293, C = 0.1435, G = 0.2114, T = 0.3159), substitution model (A–C = 0.7893, A–G = 4.7045, A–T = 1.5821, C–G = 0.8288, C–T = 4.9527, G–T = 1), and rates for variable sites following a gamma distribution (G = 4.527). BI revealed that haplotypes Belém 01 and Belém 02 are phylogenetically related to *H. canis*, while haplotype Belém 03 is phylogenetically related to *H. americanum* (Fig. 1). Pairwise comparison of 16 sequences (3 from this study and 13 retrieved from GenBank) showed p distance ranging from 0 to 6.6% (Supplementary Table 1). While the genetic distances between *H. canis* strains was estimated in 1.36% (Belém 02 × *H. canis* EF622096), the highest p distance between *H. americanum* strains was 1.37% (*H. americanum* JX415176 × *Hepatozoon* sp. KC127679).

4. Discussion

Despite optimization of the molecular method for the detection of the DNA of *Hepatozoon* spp. our protocol proved to be more efficient than that proposed by Inokuma et al. (2002), which has been broadly used for molecular detection of *Hepatozoon* spp. in dogs, but is based on a single round of standard PCR. As observed here, the protocol developed by Inokuma et al. (2002) is subject to a high frequency of false negative results, probably due to the low levels of parasitemia, i. e., the number of copies of the target DNA may be insufficient to be detected in a single round standard PCR.

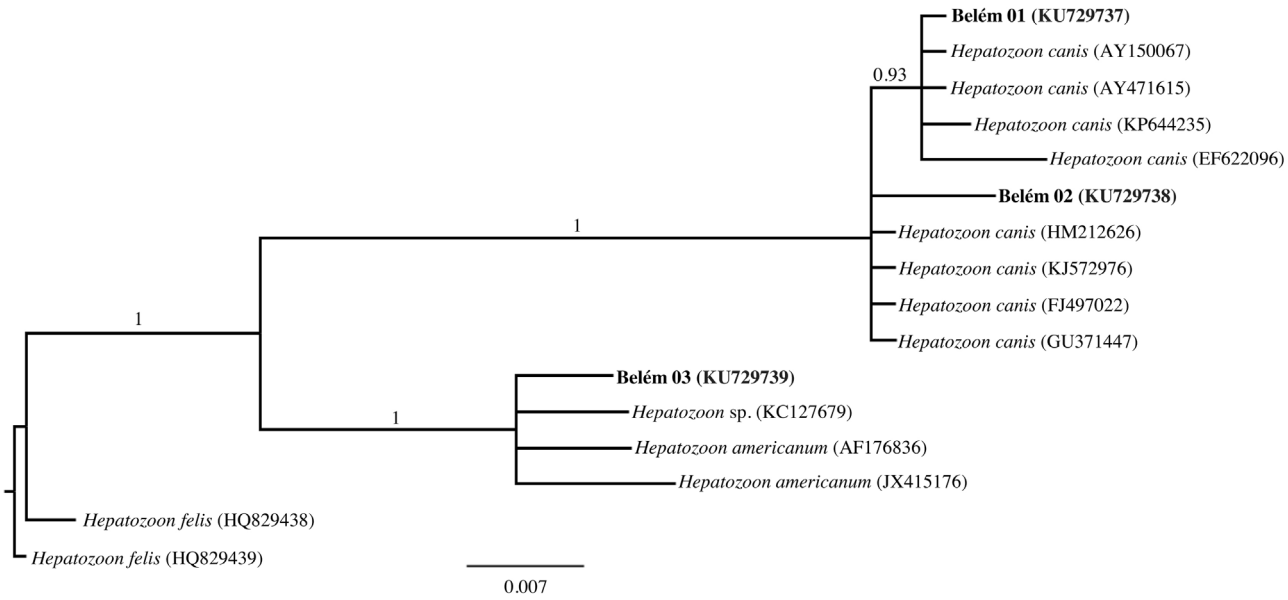

Fig. 1. Phylogenetic relationship between the partial sequences of the *Hepatozoon* 18S rRNA gene of the species found in dogs from the metropolitan area of Belém (Pará, Brazil) and species of the same genus. The numbers in the nodes indicate the value of the Bayesian posterior probability. The scale bar indicates an evolutionary distance of 0.007 nucleotides per position in the sequence. The sequences obtained in this study are in bold. Two sequences *Hepatozoon felis* were used as outgroups.

Indeed, according to O'Dwyer (2011), in Brazil, it is extremely rare to find dogs that present high level of infection by *Hepatozoon* spp. In comparison to other diagnosis methods, studies have shown that snPCR (Moraes et al., 2014), nPCR (Rufino et al., 2013) and qPCR (Criado-Fornelio et al., 2007; Souza et al., 2012) are more efficient for the detection of blood pathogens. Thus, at least in comparison to a qPCR assay, the protocol optimized in the present study represents an alternative and cheaper procedure for the detection of *Hepatozoon* spp. in dogs.

One of the most remarkable results of this study is, to the best of our knowledge, the first report of *H. americanum* naturally infecting domestic dogs in Brazil. Indeed, according to our phylogenetic analysis, since the nucleotide sequence (KC127679) obtained by Almeida et al. (2013) consistently groups with other *H. americanum* 18S rRNA sequences, our finding reinforces previous evidence that this taxon has been circulating in this country. Although Forlano et al. (2007) has suggested the possibility of occurrence of other *Hepatozoon* specie than *H. canis* in domestic Brazilian dogs, our study suggests that the detection of *H. americanum* in Brazil had been restricted to wild canids (see Almeida et al., 2013). In addition, the high identity (approximately 99%) shared by AF176836, KC127679 and AY461377 sequences do not corroborate the findings obtained by Criado-Fornelio et al. (2006), which suggests that the Brazilian and North American *H. americanum*-related protozoans do not belong to the same species. The genetic distance levels observed in this study to the *H. americanum* cluster are even similar to those we observed between some strains in the *H. canis* cluster, which are not recognized as different species (see Criado-Fornelio et al., 2006; Forlano et al., 2007).

Since there are no reports of *A. maculatum* in Brazil, one of the 61 species of ticks reported for this country (Dantas-Torres et al., 2009) is certainly not refractory to *H. americanum* infection. Nevertheless, the *H. canis* DNA was recently detected in *A. cajennense* sensu lato (Melo et al., 2016), which should be investigated as a possible vector of *H. americanum* (Ewing and Panciera, 2003).

We also found that haplotypes Belém 01 and Belém 02, which were grouped in *H. canis* cluster, present genetic distance similar to that observed for *H. canis* strains occurring in different geographical regions. Furthermore, since Belém 01 sequence is identical to *H. canis* 18S rRNA sequences found circulating in Spain (Criado-Fornelio et al., 2003) and Brazil (Criado-Fornelio et al., 2006), Belém 02 represents new data concerning the global diversity of *H. canis*. Thus, the present study suggests that there are at least two *H. canis* strains circulating among dogs is Brazil.

Notwithstanding the relatively small sample size of the present study, the prevalence of canine hepatozoonosis in Belém appears to be lower than that observed for other localities. In Brazil, based on molecular data, the prevalence of *H. canis* infection in dogs is widely variable. In Pernambuco State was found 0.49% (Ramos et al., 2010), while in Mato Grosso do Sul and in Espírito Santo States the prevalences were estimated in 3.63% (Ramos et al., 2015) and 58.7% (Spolidorio et al., 2009), respectively. Likewise, the prevalence of *H. canis* worldwide also varies considerably. Prevalence in Turkey was 3.61% (Aydin et al., 2015), with 11.4% in Thailand (Jittapalapong et al., 2006), 20.3% in Nigeria (Sasaki et al., 2008), 42.9% in Japan (El-Dakhly et al., 2013) and 42.3% in Sudan (Oyamada et al., 2005). An epidemiological survey of *H. americanum* infection in dogs, in which a molecular tool was used, revealed 10% of dogs infected in Virginia, 28.5% in Georgia, 30.9% in Alabama and 41% in Mississippi (Li et al., 2008).

Overall, the molecular procedures used in this study allowed the detection of *H. canis* and *H. americanum* naturally infecting domestic dogs in Belém, as well as their preliminary genetic diversity. The proposed method should be useful on further molecular epidemiological surveillance as well as to investigate the prevalence of canine hepatozoonosis in areas where it is undetected currently.

Conflict of interest statement

No competing financial or non-financial interests exist.

Acknowledgements

ECG is grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for individual support (grant 311686/2015-0). LAG acknowledges a doctoral scholarship from Conselho de Aperfeiçoamento de Pessoal (CAPES).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ttbdis.2016.09.008.

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