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Molecular analysis of Bartonella spp. in liver tissue of bats from the Atlantic Forest biome, Brazil

Análise molecular de Bartonella spp. em tecido hepático de morcegos do bioma Mata Atlântica, Brasil

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Highlights

Bartonella in neotropical bats.
Bartonella in liver tissue of bats.
Molecular detection of Baronella.

Abstract

The genus *Bartonella* comprises gram-negative bacilli that possess tropism for erythrocytes and endothelial cells in animals of the orders Rodentia, Lagomorpha, Carnivora, Artiodactyla, Eulipotyphla, and Chiroptera. Bacterial infection may be associated with lymphadenitis, endocarditis, bacillary angiomatosis, and peliosis hepatis. Thirteen species of *Bartonella* are recognized as zoonotic and bats are considered to be their potential reservoirs. This study aimed to analyze the occurrence of *Bartonella* spp. in livers of neotropical bats belonging to the families Molossidae, Phyllostomidae, and Vespertilionidae, classified into 21 genera from the Atlantic Forest biome in São Paulo. A total of 341 (n = 341) chiropterans samples were tested for the presence of citrate synthase (*gltA*) gene of *Bartonella* by partial amplification using polymerase chain reaction (PCR). Samples of two bats (0.6%) of the species *Glossophaga soricina*

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from the municipality of São Roque were tested positive for *gltA* gene. Phylogenetic analysis indicated that sequences clustered in a clade that was close to the *Bartonella* sp. detected in *G. soricina*, which was collected from the Cerrado biome in Tocantins, Brazil. Despite a low prevalence of the detected infection, results indicated that neotropical bats from the Atlantic Forest were potential hosts of Bartonella spp., which might be related to the maintenance of a wild enzootic cycle of the bacterium. Additional studies, particularly on bats of genus *Glossophaga,* are required to elucidate the dynamics of intraspecific relationships between etiologic agent-vector-hosts.
Key words: Neotropical bats. Liver. Bartonellaceae.

Resumo

O gênero *Bartonella* compreende bacilos gram-negativos que possuem tropismo por eritrócitos e células endoteliais em animais das ordens Rodentia, Lagomorpha, Carnivora, Artiodactyla, Eulipotyphla e Chiroptera. A infecção bacteriana pode estar associada a linfadenite, endocardite, angiomatose bacilar e peliose hepática. Treze espécies de Bartonella são reconhecidas como zoonóticas e os morcegos são considerados seus potenciais reservatórios. Este estudo teve como objetivo analisar a ocorrência de *Bartonella* spp. em fígados de morcegos neotropicais pertencentes às famílias Molossidae, Phyllostomidae e Vespertilionidae, classificados em 21 gêneros do bioma Mata Atlântica em São Paulo. Um total de 341 (n = 341) amostras de quirópteros foram testados para a presença do gene da citrato sintase (*gltA*) de Bartonella por amplificação parcial usando reação em cadeia da polimerase (PCR). Amostras de dois morcegos (0,6%) da espécie *Glossophaga soricina* do município de São Roque foram testadas positivas para o gene *gltA.* A análise filogenética indicou que as sequências agrupadas em um clado próximo ao de *Bartonella* sp. detectada em *G. soricina,* que foi coletada no bioma Cerrado no Tocantins, Brasil. Apesar da baixa prevalência da infecção detectada, os resultados indicaram que os morcegos neotropicais da Mata Atlântica eram potenciais hospedeiros de *Bartonella* spp., o que pode estar relacionado à manutenção de um ciclo enzoótico selvagem da bactéria. Estudos adicionais, particularmente em morcegos do gênero *Glossophaga,* são necessários para elucidar a dinâmica das relações intraespecíficas entre agente etiológico-vetor-hospedeiro.
Palavras-chave: Morcegos neotropicais. Fígado. Bartonellaceae.

Introduction

The genus *Bartonella* represents gram-negative bacilli belonging to the alpha-2 subgroup of the phylum Proteobacteria, which possess tropism for erythrocytes and endothelial cells (Angelakis & Raoult, 2014; Okaro et al., 2017). Among the 45 species of *Bartonella* recognized, the vast majority are described in wild animals belonging to the orders Rodentia, Lagomorpha, Carnivora, Artiodactyla, Eulipotyphla, and Chiroptera (Saisongkorh et al., 2009; Okaro et al., 2017).

In humans, 13 zoonotic or potentially zoonotic *Bartonella* spp. are associated with syndromes with varied clinical manifestations, including cat-scratch disease (*B. henselae*), lymphadenitis (*B. clarridgeiae*), fever associated with persistent bacteremia (*B. Rochalimae, B. henselae, B. quintana,* and *B. tamiae*), diseases such as trench fever (*B. quintana*), Peruvian wart (*B. ancashensis, B.*

bacilliformis), Oroya fever (*B. bacilliformis*), neuroretinitis lesions (*B. grahamii*), endocarditis (*B. alsatica, B. elizabethae, B. henselae, B. koehlerae, B. mayotimonensis,* and *B. vinsonii*), and angioproliferative lesions such as bacillary angiomatosis/peliosis hepatis (*B. henselae* and *B. quintana*) (Kordick & Breitschwerdt, 1997; Okaro et al., 2017).

The reservoirs and transmission routes of many *Bartonella* spp. remain undetermined. However, both in natural infection and that simulated by experimental studies, hematophagous arthropod vectors may participate in the transmission of *Bartonella* spp. between animals, which suggests that invertebrates act as potential dispersers of the pathogen to humans (Breitschwerdt, 2017).

Bats (Order: Chiroptera) contribute to the maintenance of the epidemiological cycle of bartonellosis in humans, as *Bartonella mayotimonensis,* which causes endocarditis in humans, has already been detected in bats in Finland (Okaro et al., 2017). Other *Bartonella* spp. were detected in China, France, Spain, Romania, Brazil, and Japan (Stuckey et al., 2017; Corduneanu et al., 2018; Nabeshima et al., 2020; Gonçalvez-Oliveira et al., 2020).

Chiropterans harbor an array of *Bartonella* spp. favoring the maintenance of bartonellosis cycle (Pacheco et al., 2010; Okaro et al., 2017). Molecular techniques have aided to investigate *Bartonella* infection in blood, tissue, and bacterial culture samples from bats of multiple species, those from different geographic regions, and those indicating varying prevalence rates (Kosoy et al., 2010; Angelakis & Raoult, 2014; Okaro et al., 2017; Gonçalvez-Oliveira et al., 2020). In Latin America, isolates of *Bartonella* spp. have been identified in bats captured in tropical forest fragments in Conguaco, Oratorio, San Lucas Tolimán, Santa Lucía Cotzumalguapa, Taxisco in Guatemala (Bai et al., 2011), in forest fragments of the Amazon biome in Peru (Bai et al., 2012), and in the urban city of Buenos Aires in Argentina (Cicuttin et al., 2017).

In Brazil, isolates of *Bartonella* spp. were described in bats from the Atlantic Forest fragments (Ikeda et al., 2017; Ferreira et al., 2018; Gonçalvez-Oliveira et al., 2020), Cerrado and the Amazon biome; however, data on the transmission of *Bartonella* spp. among bats and other wild mammals are still scarce, despite the importance of the bacterium in public health (Ikeda et al., 2017). This study aimed to evaluate the prevalence of *Bartonella* spp. infection and their phylogenetic characteristics in liver tissue samples of neotropical bats from São Paulo.

Material and Methods

The retrospective cross-sectional study evaluated 341 (n = 341) bat liver samples from the Atlantic Forest biome in São Paulo, which were stored at -70°C in the specimen bank of the Hepatology Section of Instituto Evandro Chagas (IEC). Samples were obtained through tissues undergoing necropsy in bats that were sent to the Pasteur Institute (State Health Department, Government of São Paulo) by 90 municipalities in São Paulo during the years 2012, 2014, and 2015 for epidemiological surveillance of rabies. This research was reviewed and approved by the Ethics Committee on the Use of Animals of the IEC (protocol: n° 033/2017).

Liver samples of 341 bats corresponded to three families (Molossidae, Phyllostomidae, and Vespertilionidae) and 21 species (Table 1). Bats were identified with the help of specific taxonomic keys such as that of Reis et al. (2010).

Table 1
Distribution of neotropical bats into families, species, and sex

Chiroptera family	Bat species	Number of specimens	
		Females	Males
Phyllostomidae	*Desmodus rotundus*	9	7
	Glossophaga soricina	28	25
	Anoura caudifer	-	1
	Carollia perspicillata	-	1
	Artibeus fimbriatus	-	1
	Platyrrhinus spp.	-	1
	Platyrrhinus lineatus	3	5
	Sturnira lilium	6	5
	Artibeus lituratus	28	23
Molossidae	*Cynomops greenhalli*	1	
	Cynomops planirostris	3	6
	Eumops auripendulus	2	1
	Eumops glaucinus	23	14
	Eumops perotis	3	6
	Molossus molossus	24	62
	Molossus rufus	2	19
	Nyctinomops laticaudatus	1	2
	Tadarida brasiliensis	1	1
Vespertilionidae	*Lasiurus egra*	2	-
	Histiotus spp.	-	1
	Histiotus velatus	-	1
	Myotis nigricans	8	6
	Eptesicus furinalis	2	7
TOTAL		146	195

Total DNA was purified from each liver sample using the DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions. DNA was eluted in a volume of 200 µL and stored at -70°C until molecular analysis. PCR assays were developed under previously described conditions (Birtles & Raoult, 1996; Cicuttin et al., 2017) for

partial amplification of the Bartonella spp. gltA gene (350 bp). Partial amplification and phylogenetic analysis of the gltA gene has demonstrated adequate sensitivity and specificity for molecular identification of Bartonella spp. (Birtles & Raoult, 1996; Gutiérrez et al., 2017).

The GoTaq® Hot Start Polymerase kit (PROMEGA Corporation, Madison, USA) was used for PCR by adding primers CSH1f (5'-GCGAATGAAGCGTGCCTAAA-3') and BhCS.l137n (5'-AATGCAAAAAGAACAGTAAACA-3') (Birtles & Raoult, 1996; Gutiérrez et al., 2017) and making a reaction of 25 µL, which was adjusted according to the manufacturer's guidelines. The thermocycling program followed the time and temperature conditions described by Bai et al. (2011) and Norman et al. (1995).

Amplicons were detected by electrophoresis in 1% agarose gel with the aid of a UV transilluminator. Samples with amplification products approximately 350 bp in size were considered positive (Norman et al., 1995; Birtles & Raoult, 1996; Gutiérrez et al., 2017). Total DNA aliquots of wild rodent liver positive for Bartonella spp. and DEPC water were added as positive and negative controls for PCR reactions, respectively.

Amplicons from positive samples were enzymatically purified (ExoSAP-IT, GE Healthcare) and sequenced in forward and reverse directions on an automated sequencer AB 3500 Genetic analyzer (Thermo Fisher Scientific, Waltham, MA, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and BigDye XTerminator Purification Kit (Thermo Fisher Scientific), according to the manufacturer's guidelines.

Sense and antisense sequences were aligned using Geneious v8.1.3 software to obtain consensus sequences, which were submitted to BLASTn (www.ncbi.nlm.nih.gov/blast/Blast.cgi) to confirm Bartonella spp. positivity. Consensus sequences of positive samples were deposited to the GenBank platform (http://www.ncbi.nlm.nih.gov/genbank) and aligned with a database containing 167 nucleotide sequences representative of the genus Bartonella available in the GenBank database for phylogenetic analysis by maximum-likelihood method (GTR+G+I) using MEGA v7.0 software (1000 bootstrap replicates) and taxonomic identification of the isolates. The nucleotide distance was calculated using Geneious v8.1.3 software.

Results and Discussion

Results of molecular tests and phylogenetic analyses indicated that two (0.6%, 2/341) bat liver samples were positive for Bartonella spp. These positive samples belonged to Glossophaga soricina bats, both from the municipality of São Roque in São Paulo.

In the phylogenetic analysis, the nucleotide sequences obtained from the two samples (GenBank accession numbers MG878887 and MG878888) grouped together in a clade close to a Bartonella sp. obtained from the bat G. soricina from Tocantins, Brazil (Figure 1).

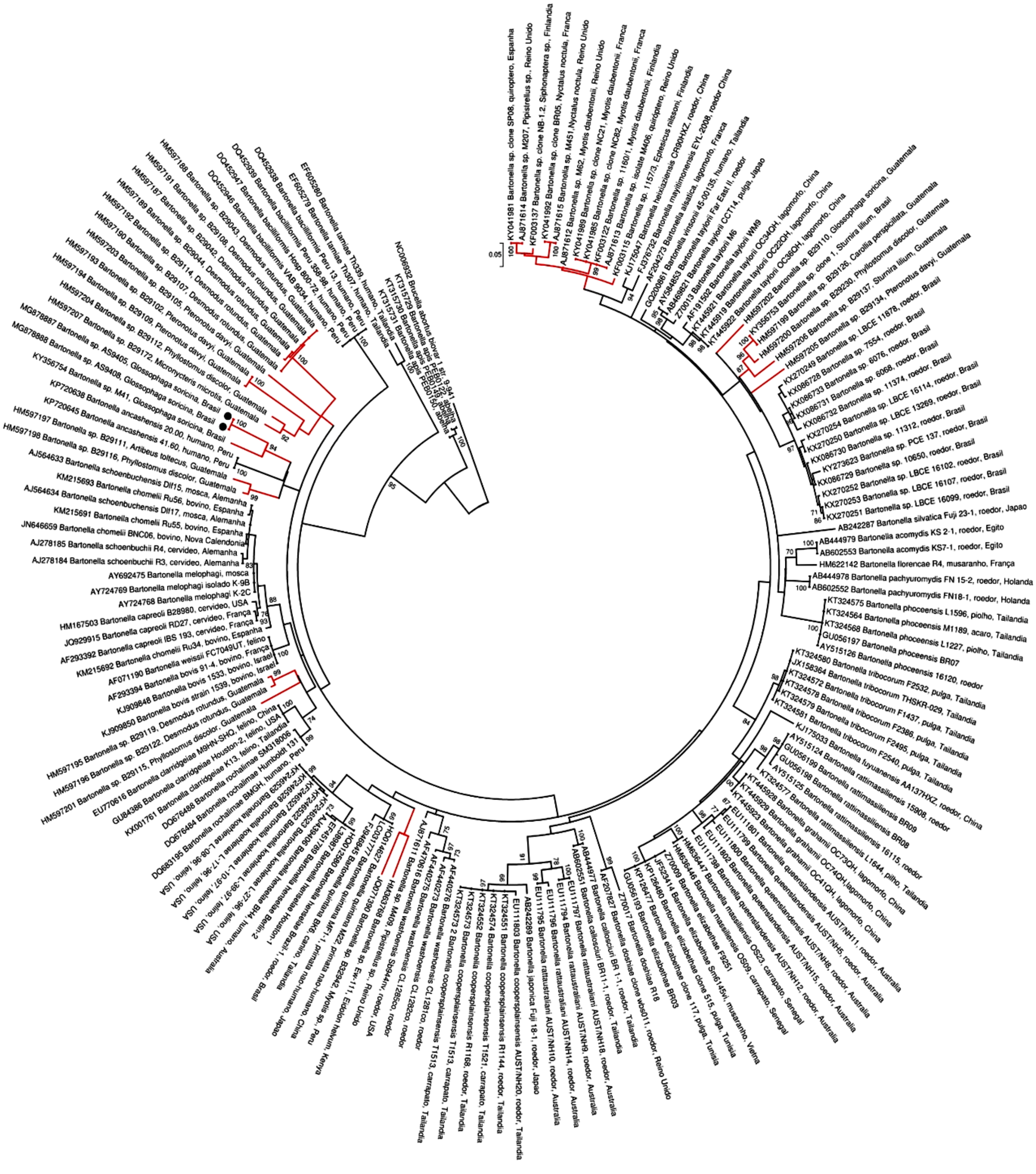

Figure 1. Phylogenetic tree of the genus *Bartonella* inferred by the maximum likelihood method, based on partial nucleotide sequences (243 bp) of the *gltA* gene. The sequences are identified by the GenBank accession code, identification of species/isolates, hosts, and geographical origin. Positive samples from the present study are highlighted (●) and branches containing sequences obtained from bats are marked in red. A sequence from *Brucella abortus* (NC006932) was used as an outgroup. Boostrap values > 70 are expressed in nodes. Slash represents nucleotide substitutions per site.

Sequences obtained from the two *G. soricina* bats showed 100% nucleotide identity among themselves. When compared with other partial sequences of the *gltA* gene obtained from bats in the Old and New World, the nucleotide identity range was 82.1–89.8%.

The two Bartonella spp. detected in G. soricina in the present work showed different percentages of nucleotide identity with *Bartonella* spp. from other species - 82.1% with the isolate identified in *Pteronotus davyi* in Guatamela (Bai et al., 2011), 82.5% with the isolate recorded in *G. soricina* in Tocantins, Brazil, and 83.2% with *Bartonella* sp. identified in *Stunira lilium* in Paraná (André et al., 2019). Compared with other representative sequences of *Bartonella*, the percentage of nucleotide identity of the two positive samples in the study was 86% with those of *B. ancashensis,* 84.6% with those of *B. henselae,* 81.4% with those of *B. bacilliformis,* and 82 .9% with those of *B. mayitimonensis.*

Despite the low prevalence of *Bartonella* spp. infection among neotropical bats identified in the present study, the result represented the detection of *Bartonella* sp. in *G. soricina* (Phyllostomidae) bats from the Atlantic Forest biome in São Paulo for the first time.

The bat *G. soricina* belongs to the family Phyllostomidae and subfamily Glossophaginae, which includes bats with adaptations to nectarivory exploring the flora of the understory. It has a wide geographic distribution in countries such as Mexico, Panama, Peru, Costa Rica, Bolivia, Costa Rica, and Brazil. In Brazil, it has already been recorded in different states including São Paulo (Reis et al., 2010).

In this study, *G. soricina* was the only species that detected positive for *Bartonella* spp. infection. As this species has synanthropic habits and uses houses and buildings as shelter and to look for food, these factors can strengthen interspecific relationships with humans and other animals (Reis et al., 2010). Such niche exploration behaviors may favor a potential for the transmission of bacteria from the wild to domestic and peri-domestic areas (Ferreira et al., 2018; Gonçalvez-Oliveira et al., 2020) and possible establishment of an anthropozoonotic cycle (Pacheco et al., 2010).

Ikeda et al. (2017) identified a 5.2% prevalence of *Bartonella* spp. among bats of the species *G. soricina* in Tocantins, *Sturnira lilium* in Paraná, *Carollia perspicillata, Phyllostomus discolor,* and *Natalus espiritosantensis* in Pará State. Notably, in this study, isolates of Bartonellaceae identified in *G. soricina* also revealed phylogenetic similarity with the monophyletic clade from Costa Rica described for the same bat species.

Olival et al. (2015) identified a direct proportional relationship between the prevalence of *Bartonella* spp. and the load of ectoparasites in bats from Puerto Rico. We did not evaluate the correlation between presence of ectoparasites and the occurrence of *Bartonella* spp. infection; however, studies that evaluate the relationship between parasite and host, such as the works by Amaral et al. (2018) and Gonçalves et al. (2020), can provide relevant information on the bartonellosis cycle between bats and other wild mammals in Brazil.

Currently, direct amplification molecular tests have been used on a large scale to investigate *Bartonella* spp. in viscera (Gonçalvez-Oliveira et al., 2020), blood (Matthew et al., 2017), and bacterial cultures (Billeter et al., 2012).

The choice of liver tissue for detection of Bartonellaceae is related to its cycle in the host liver as one of the organs of bacterial tropism (Boulouis et al., 2005; Muhldorfer, 2012).

Chiropterans are the important hosts for different *Bartonella* spp. genotypes (Ikeda et al., 2017; Stuckey et al., 2017; Ferreira et al., 2018), a characteristic that may be related to the fact that the order Chiroptera has species with wide geographic distribution, ease of movement, social behavior, and niche exploratory behavior (Reis et al., 2010). Previous studies identified *Bartonella* spp. in liver samples from *Artibeus lituratus, Carollia perspicillata* and *Desmodus rotundus* bats, with insectivorous, frugivorous, folivorous, and hematophagous feeding habits (André et al., 2019; Gonçalvez-Oliveira et al., 2020), and in this study, in nectivorous bats, suggesting diverse natural transmission cycles of the bacterium in neotropical bats in Brazil.

Quantitative PCR (qPCR) method has higher sensitivity for *Bartonella* spp. detection when compared with conventional PCR (Kamani et al., 2014). The *Bartonella* research strategy by direct amplification (without bacterial culture) from chiropteran tissues samples, based on partial amplification of the *gltA* gene as used in the present study, has already been described and validated by Kosoy et al. (2010) in Kenya, Dietrich et al. (2016) in Southern Africa, and Brook et al. (2015) in Madagascar.

Ikeda et al. (2017) observed parasite-host relationship specificity, as they identified the *Bartonella* genotype in *G. soricina* in Tocantins similar to that in bats of the same species from Costa Rica. As the *Bartonella* spp. isolates infecting *G. soricina* bats in this study showed high nucleotide identity with those identified in the same bat species in the state of Tocantins (Ikeda et al., 2017), this finding may corroborate the hypothesis of circulation of specific genotypes between different bat species.

In the context of emergence or re-emergence of zoonoses from pathogens originally described among bats (Stukey et al., 2017; Rahman et al., 2020), research related to surveying the health status of neotropical bats should be encouraged to analyze the *Bartonella* spp. flow of dispersal and diversity in the Brazilian territory (Ikeda et al., 2017; Ferreira et al., 2018; André et al., 2019; Gonçalvez-Oliveira et al., 2020). This should be done especially in animals from forest fragments of the Atlantic Forest, as these areas observe intense anthropogenic driven loss of the natural habitat of bats. This increases the possibility of human beings coming into contact with a sylvatic enzootic cycle such as that of bartonellosis.

Conclusion

In conclusion, the occurrence of a *Bartonella* spp. wild enzootic cycle is possible in neotropical bats captured in the Atlantic Forest biome in the municipality of São Roque, which, despite the low frequency of the isolated genotypes, may be specific for *Glossophaga* spp., hence corroborating previous records in São Paulo. The detection of

infection to other wild animals that share the same habitat as that of these bats should be expanded, which allows for delineation of the cycle, geographic dispersion, and additional taxonomic characterization of *Bartonella* spp. isolates. There is a wide variety of hosts and an interspecific relationship between the etiological agent and host, which provide a better ecoepidemiological characterization of bartonellosis in the Brazilian territory and its importance in public health.

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Conflict of interest declaration

The authors declare no conflict of interest. Funding sponsors had no role in the study design, in the collection, analysis or interpretation of data, in the writing of the manuscript, and in the decision to publish the results.

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