Occurrence and diversity of Sarcocystidae protozoa in muscle and brain tissues of bats from São Paulo state, Brazil

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A B S T R A C T
Studies on infectious and emerging diseases caused by bats have been increasing worldwide due to their well-recognised status as a reservoir species for various infectious agents as well as their close relationship to humans and animals. This study reports the molecular frequency and diversity of the parasites belonging to the Sarcocystidae family in bats in São Paulo state, Brazil. A total of 2892 tissue samples (brain and pectoral muscle/human homogenates) from 1921 bats belonging to 36 species were collected, and the Sarcocystidae protozoan 18S ribosomal RNA encoding genes (18S rDNA) were detected by nested PCR and Sanger sequencing. The relative prevalence of Sarcocystidae species was 4.7% (91/1921) among 16 bat species, including insectivorous (n = 65), frugivorous (n = 13) and nectarivorous (n = 11) bats. From 66 sequenced positive samples, 50 were found to be suitable for analysis. Ten samples from insectivorous and nectarivorous bats showed 100% similarity with Neospora caninum (n = 1), Hammondia hammondi (n = 1), Cystoisospora canis (n = 1), Nephroisospora eptesici (n = 1), Sarcocystis (Frenkelia) glareoli (n = 1), and Toxoplasma gondii (n = 5). The 45 non-T. gondii samples revealed 15 different 18S rDNA alleles with identities varying from 96.1 to 100% with several Sarcocystidae species, which might suggest that bats can harbour a large variety of Sarcocystidae organisms. From the five T. gondii-positive tissue samples, three samples from two different bat specimens of the insectivorous Eumops glaucus were characterised using 11 PCR-restriction fragment length polymorphism (RFLP) markers, revealing the non-archetypal ToxoDB genotypes #6 (type Br1), which is one of the most prevalent in different hosts and regions from Brazil, and #69. We recommend the inclusion of T. gondii as a differential diagnosis for rabies and other neurological syndromes in bats.

1. Introduction
In Brazil, bats inhabit several urban areas including bridges, building overhangs, brick houses, culvert pipes, abandoned quarries, building expansion joints, construction tent, grills, and air conditioners (Reis et al., 2002). They have various predators such as owls, hawks, eagles, falcons, raccoons, cats, snakes, toads, and large spiders. Certain bats may eat other bats, however they are generally not considered cannibals because they prey on species other than their own (Fenton and Ratcliffe, 2010). Bat diet differs from species to species; they can be carnivorous, frugivorous, insectivorous, omnivorous, hematophagous, and even nectarivorous (Ferrarezi and Gimenez, 1996). Bats also serve as a food source for some human populations in the Pacific islands, South-East Asia, Madagascar, and some native tribes in Brazil and in Africa...
Environmental changes due to urban development may have contributed to the increase of the bat population in urban areas, not only because of the wide variety of shelters but also because of the large food supply (Sodre et al., 2010).

Bats are reservoirs of several infectious diseases. Due to their diversity, worldwide distribution, and their proximity to people and domestic animals, their potential to transmit zoonotic diseases has been increasing (Bessa et al., 2010; Calisher et al., 2006; Muhldorfer et al., 2011). Infectious agents transmitted by bats include viruses, bacteria, fungi, and parasites. Transmission of these agents among bats can occur through infected saliva delivered via bites and licking, inhalation of aerosols via infected saliva, urine, or guano, ingestion of regurgitated infected blood in vampire bat, and probably by ingestion of contaminated insects, fruits, or water (Constantine, 1988; Souza et al., 2009).

Parasites from the Sarcocystidae family, Apicomplexa phylum, are of special importance since bats can serve as natural intermediate hosts. The Sarcocystidae family life cycle involves carnivores as definitive hosts that can excrete oocysts in the faeces, contaminating the environment. Ingestion of intermediate host tissues containing cysts (predator-prey route), or food and water contaminated with oocysts can lead to diseases or infections, and there is also potential vertical transmission.

A recent study has focused on the detection and identification of Sarcocystis spp. in bats in São Paulo state, Brazil. The analysis of the partial 18S rDNA sequences revealed that ten of 29 samples were positive for prevalence data. The results presented here are not correlated with the previously published studies by Cabral et al. (2013) and Cabral et al. (2014) on the isolation and seroprevalence of T. gondii in bats, but both concern bats from the São Paulo state and samples were collected during the same period.

### 3. Results

#### 3.1. Molecular detection of Sarcocystidae species in brain and heart/pectoral muscle of bats

A total of 2892 bat tissue samples were analysed. It was found that 94 samples (3.2%) were positive for Sarcocystidae protozoa; 68 (3.8%) were from brain samples and 26 (2.3%) were from heart/pectoral muscle samples. Two bats were positive in the brain and muscle tissues among 57 positive specimens that had both brain and muscles examined. A prevalence of 4.7% (91/2121) was observed among 16 bat species, including 11 insectivorous (n = 65), four frugivorous (n = 13) and one nectarivorous (n = 11) bats. Two positive bat specimens could not be classified, and in the case of positive heart/pectoral muscle specimen pools and negative individual brains, just one animal was considered as positive for prevalence data (Supplementary Data S1, Table S1D). From the 66 sequenced samples, 50 were selected for further analysis using the BigDye Terminator v.3.1 Cycle Sequencing Kit according to the manufacturer’s instructions. The sequencing was carried out using 3730 or 3100 DNA Analyzer (Applied Biosystems, USA).

The contig assembly of the sequences obtained was evaluated with Phred/Phrap/Consed from CodonCode Aligner software (Ewing and Green, 1998; Ewing et al., 1998). The identification of the nPCR-18S rDNA final sequences was performed using Basic Local Alignment Search Tool (BLAST) available in the National Center for Biotechnology Information (NCBI; https://blast.ncbi.nlm.nih.gov/). DNA of tissue samples presenting 100% identity sequence with T. gondii was submitted for PCR- restriction fragment length polymorphism (RFLP) genotyping, as previously described (Su et al., 2010) using the genetic markers SAG1-SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico, and CS3 (Pena et al., 2008).

The results presented here are not correlated with the previously published studies by Cabral et al. (2013) and Cabral et al. (2014) on the isolation and seroprevalence of T. gondii in bats, but both concern bats from the São Paulo state and samples were collected during the same period.
highest similarity with the corresponding sequences of 18S rRNA of the Sarcocystidae species available in GenBank and were calculated to range from 96.1% to 98.7%.

The protozoa *T. gondii*, *N. caninum*, *H. hammondi*, *S. (Frenkelia)* glareoli, *C. canis*, *C. belli*, and *H. lieberkuehni* were associated with bat species that can cohabit and inhabit indoor shelters. *Besnoitia besnoiti* was associated with bat species that prefer outdoor shelters while *N. epitesici* was associated with bat species inhabiting indoor and outdoor shelters. Examining the trophic group, samples from insectivorous bats were close to all sarcocystids found, except for *B. besnoiti*, which was associated with a frugivorous species. Samples close to *N. epitesici* were associated with five frugivorous species, four insectivorous species and one nectarivorous one. The zoonotic *T. gondii* protozoan was associated with insectivorous and nectarivorous bats.

### 3.2. *T. gondii* genotyping

Five bat tissues were positive for *T. gondii*, corresponding to four animals and three different species (Table 1). Two non-archetypal genotypes were elucidated from two different specimens of the *Eumops glaciatus* insectivorous bat: ToxoDB-RFLP non-archetypal genotypes #6 and Apico markers, respectively.

### Table 1

Molecular identity of Sarcocystidae 18S ribosomal RNA (rRNA) nested-PCR fragment amplified from muscle and brain tissues of different bat species collected in municipalities from São Paulo state, Brazil.

| Sample | Bat species | Trophic group | Municipality                  | Molecular identity (GenBank) |
|--------|-------------|---------------|--------------------------------|------------------------------|
| M489   | Molossus molossus | Insectivorous | São Paulo                     | 96.1% *T. gondii* (MN595284)  |
| M494*  | Molossus molossus | Insectivorous | São Paulo                     | 97% *T. gondii* (MN595284)    |
| M500   | Molossus molossus | Insectivorous | São Paulo                     | 98.7% *T. gondii* (MN595284)  |
| M520   | Molossus molossus | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M826   | Molossus molossus | Insectivorous | São Paulo                     | 98.2% *T. gondii* (MN595284)  |
| M865   | Glossophaga soricina | Nectarivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M1109* | Molossus molossus | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B454   | Molossus molossus | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B44    | Molossus molossus | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B1345  | Molossus molossus | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B1349  | Molossus molossus | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M48,B651 | Eumops glaciatus         | Insectivorous | Piracicaba                | 96.1% *T. gondii* (MN595284)  |
| B1466  | Eumops glaciatus         | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M60    | Molossus molossus | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M1052  | Glossophaga soricina | Nectarivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M774   | Molossus molossus | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M853   | Platyrhinus lineatus | Frugivorous  | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B265   | Artibeus planirostris    | Frugivorous  | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B649   | Artibeus lituratus       | Frugivorous  | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B688   | Artibeus lituratus       | Frugivorous  | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B955   | Artibeus lituratus       | Frugivorous  | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B1002  | Glossophaga soricina | Nectarivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B1398  | Artibeus lituratus       | Frugivorous  | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B1682  | ND                       |               | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B1700  | Artibeus lituratus       | Frugivorous  | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B1707  | Artibeus lituratus       | Frugivorous  | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B1740  | Phyllostomus discolor    | Frugivorous  | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M886   | Platyrhinus lineatus     | Frugivorous  | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B1397  | Gymnophia planirostris   | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M1342  | Myotis nigricans         | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M1121  | Histiotus velatus        | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B789   | Eptesicus furinalis      | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M493*  | Molossus molossus        | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M496*  | Nyctinomops macrotis     | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M496*  | Nyctinomops macrotis     | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M528   | Nyctinomops macrotis     | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B657   | Nyctinomops laticaudatus | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B829   | Nyctinomops laticaudatus | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B999   | Molossus molossus        | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M1148  | Glossophaga soricina     | Nectarivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B935   | Artibeus lituratus       | Frugivorous  | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M437   | Artibeus lituratus       | Frugivorous  | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B332   | Glossophaga soricina     | Nectarivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M504   | Molossus molossus        | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| M222,B468 | Tadarida brasiliensis      | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |
| B159   | Tadarida brasiliensis     | Insectivorous | São Paulo                     | 97% *N. epitesici* (EU334134) |

M: muscle; B: brain; ND: not determined; *: DNA extracted from more than one bat specimen (tissue pool).
Table 2
Genotyping of Toxoplasma gondii from tissue samples in bats from São Paulo state, Brazil, by multilocus PCR-restriction fragment length polymorphism (PCR-RFLP).

| PCR-RFLP markers | Identity with other species | Bat species | Sample | Host | Identity with other isolates |
|------------------|----------------------------|-------------|--------|------|-----------------------------|
| SAG1             |                            | Rattus rattus | #69    | Chicken | SAG2, SAG3, and other isolates from chickens from Brazil (Dubey et al., 2000; Dubey et al., 2004; Dubey et al., 2011; Dubey et al., 2012) |
| SAG3             |                            | Eumops      | M48    | Chicken | SAG2, SAG3, and other isolates from chickens from Brazil (Dubey et al., 2000; Dubey et al., 2004; Dubey et al., 2011; Dubey et al., 2012) |
| SAG2             |                            | Nyctinomops | M48    | Chicken | SAG2, SAG3, and other isolates from chickens from Brazil (Dubey et al., 2000; Dubey et al., 2004; Dubey et al., 2011; Dubey et al., 2012) |
| E. glaucinus     |                            | Rhinolophus | B651   | Chicken | SAG2, SAG3, and other isolates from chickens from Brazil (Dubey et al., 2000; Dubey et al., 2004; Dubey et al., 2011; Dubey et al., 2012) |
| B. hypolophus    |                            | Nyctinomops | B63    | Chicken | SAG2, SAG3, and other isolates from chickens from Brazil (Dubey et al., 2000; Dubey et al., 2004; Dubey et al., 2011; Dubey et al., 2012) |
| B. hypolophus    |                            | Nyctinomops | B63    | Chicken | SAG2, SAG3, and other isolates from chickens from Brazil (Dubey et al., 2000; Dubey et al., 2004; Dubey et al., 2011; Dubey et al., 2012) |

Discussion

In the present study, detection of Sarcocystidae protozoa directly from muscle and brain tissues of bats was successfully performed through PCR amplification of a conserved 18S rRNA encoding sequence common to T. gondii, N. caninum, H. hammondi, N. eptesici, and S. neurona protozoa (Su et al., 2010).

Except for the samples identified as T. gondii, confirmed as such by other molecular markers, the other candidate sarcocystids found in bats could not be accurately identified because the short 18S rRNA coding segment has little discriminatory value for unequivocal species identification. The identification of sarcocystids, especially within the genus Sarcocystis, must be based on multilocus analyses using barcoding markers as internal transcribed spacers within ribosomal loci, genes within the mitochondrial genome, or in apicoplasts and others (Gjerde et al., 2013; Kirillova et al., 2018; Cesar et al., 2019).

Nevertheless, 15 alleles were found among the 45 partial 18S sequences that were classified as non-T. gondii organisms and showed similarity of 96.1%–100% with other sarcocystids of Sarcocystis, Toxoplasma, toxoplasmatinae, and sarcocystidae subfamilies. The 18S rDNA sequence screening suggests that bats in Brazil can harbour a large variety of Sarcocystidae organisms, even though molecular targets with higher phylogenetic resolution would be necessary to clarify these identities. Studies aiming to identify sarcocystids through molecular analysis employing other molecular markers have enormous potential to identify new species in bats, particularly if associated with morphological studies.

Herein, we report DNA similar to C. canis, S. (Frenkelia) glareoli (in the insectivoruous bats M. mosolus and N. laticaudatus, respectively), and H. hammondi (in the nectarivorous Glossophaga soricina) for the first time in bats. In addition, DNA similar to N. caninum was found in a G. soricina for the first time. Recently, a DNA fragment corresponding to the ribosomal internal transcribed spacer from N. caninum was also found in four Rhinolophus pusillus insectivoruous bats (Wang et al., 2018). These results show the importance of understanding the epidemiological chain of these protozoa in bats, the second largest globally distributed mammals with major ecological importance. The insectivoruous and nectarivarivorous bats could be infected by Sarcocystidae parasites through ingestion of oocysts present in water, nectar, or mechanically carried by insects; bats could also be infected vertically in the case of T. gondii and N. caninum (Dubey et al., 1988; Fayer et al., 2015; Donahoe et al., 2015).

Toxoplasma gondii is the most investigated parasite among the Sarcocystidae family due to its connection to human and veterinary health. Despite its high prevalence in humans and warm-blooded animal populations, only a small percentage of infected individuals exhibit clinical symptoms, thus demonstrating variability (Dubey, 2010; Gilbert et al., 1999). In order to identify factors associated with variable clinical characteristics of toxoplasmosis, genotyping of T. gondii obtained from animals and humans have been performed around the world (Shaw et al., 2014). Based on several studies, it was not possible to establish a clear relationship between clinical manifestations of toxoplasmosis and genotype variability. However, the T. gondii genotype profile presents differences in geographical and population structures. For example, isolated strains from Europe are predominantly type II; low genetic diversity was also observed in populations in Africa and Asia, where type II, III, and Chinese I are the most prevalent (Shaw et al., 2014). In Central and South America, presence of higher genetic diversity maybe associated with recurrent infections (Costa et al., 2018).

In this study, genotyping of T. gondii from two E. glaucinus insectivoruous bats from the countryside of São Paulo revealed non-archetypal genotypes #69 and #6. The T. gondii genotype #69 has been described only in chickens in Brazil. Thus, this is the first report in bats. Genotype #6 (type Brl or Africa 1) is widely distributed across Africa and Brazil and in different host animals, including humans (Shaw et al., 2014). However, it has been identified to circulate in Chiroptera for the first time in Brazil.
time. These results corroborate the high genetic variability of *T. gondii* in Brazil and the importance of bats as natural intermediate hosts for this zoonotic protozoan. Furthermore, toxoplasmosis can be the cause of important neurological disease in bats, as described in megachiropteran from Australia (Sangster et al., 2012), it would be advisable to include *T. gondii* as a differential diagnosis for neurological syndromes in bats.

The proximity between humans and bats in urban and rural areas is part of a broader scenario of environmental changes. Diseases can emerge as a result of new biological interactions between living species, caused by disturbance of the ecological balance. Habitat fragmentation is a dominant feature of the modern landscape (Ewers and Didham, 2006), and species response to fragmentation has cascading effects on bat communities. Bats are considered an excellent bioindicator of environmental changes caused by human activities (Jones et al., 2009).

In the present study, the importance of bats as reservoirs of Sarcocystidae parasites was investigated and it is suggested that the diagnosis of *T. gondii* should be included as differential for rabies and other neurological syndromes in this group of animals.

Declaration of competing interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2021.01.003.

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Declaration of interests

None.

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