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

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ABSTRACT

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/heart 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 glacinus* were characterised using 11 PCR-restriction fragment length polymorphism (RFLP) markers, revealing the non-archetypal ToxoDB genotypes #6 (type BrI), 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

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(Jenkins and Racey, 2008; Setz and Sazima, 1987).

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 (through tachyzoites) for the Toxoplasmatinae subfamily species (Dubey et al., 1988; Fayer et al., 2015; Donahoe et al., 2015). Bats are hosts of *Toxoplasma gondii* (Sangster et al., 2012; Cabral et al., 2013; Dodd et al., 2014); *Sarcocystis* spp. (Duignan et al., 2003; Muhldorfer et al., 2011); *Besnoitia* spp. (Hornok et al., 2015); *Neospora caninum* (Wang et al., 2018), and *Nephroisospora eptesici* (Wunshmann et al., 2010).

Nevertheless, studies on Sarcocystidae protozoan infections in bats are limited, especially the identification of species. Our study reports the molecular frequency and diversity of the important group of parasites from the Sarcocystidae family in bats from São Paulo state, Brazil, through molecular detection and sequence analysis based on the 18S rRNA genes.

2. Material and methods

2.1. Bat tissue collection and DNA extraction

From March 2010 to March 2011, 1921 bats from 13 municipalities of São Paulo, a Brazilian state, were evaluated for rabies by the São Paulo Zoonosis Control Center (CCZ-SP), as part of a passive surveillance. The locality of collection, bat classification, trophic group, and biological data (gender, age) are provided in Supplementary Data S1 (Fig. S1A; Table S1B; Table S1C). The classification of bats was performed as per the method established by Vizotto and Taddei (1973). Frozen animals, negative for rabies, were manipulated in a laminar flow hood and the brains (n = 1774), and pectoral muscle/heart homogenates (n = 1118) were aseptically collected. Subsequently, DNA extraction was performed using Wizard Genomic DNA Purification Kit (Promega, USA). All protocols used in this study were in accordance with ethical principles in animal research adopted by Scientific Committee of the CCZ-SP and Ethic Committee for the Use of Animals of the School of Veterinary Medicine and Animal Science of University of São Paulo (protocol number 1679/2009).

2.2. Sarcocystidae molecular detection, identification, and Toxoplasma gondii genotyping

Detection of Sarcocystidae parasites was performed using nested PCR (nPCR) with the amplification of a 312 bp PCR fragment encoding the 18S ribosomal RNA (18S rDNA) in the first round of PCR. For the second round of amplification (nPCR-18S rDNA), a fragment of 291 bp for *Sarcocystis neurona*, *N. caninum*, *Hammondia hammondi*, and *T. gondii,* and a fragment of 310 bp for *Sarcocystis* spp. (Su et al., 2010). The first PCR reaction was performed using 50–100 ng of total genomic DNA, 25 μM of the external primers Tg18s48F (5′ CCATGCATGTCTAAGTATAAGC 3′) and Tg18s359R (5′ GTTACCCGTCACTGCCAC 3′), 200 mM of dNTPs, 1.5 mM of $MgCl_2$, 5 mM of KCl, 1 mM of Tris-HCl (pH 9.0), and 0.5 U of proofreading Taq DNA Polymerase. For nPCR-18S rDNA, the same reagents were used with a total volume of 50 μL, 2 μL of the first PCR reaction diluted with ultra-pure water (1:2), and 50 μM of the internal primers Tg18s58F (5′ CTAAGTATAAGCTTTTATACGGC 3′) and Tg18s348R (5′ TGCCACGGTAGTCCAATAC 3′). The cycling conditions for the first PCR reaction were 30 cycles of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 120 s. The same for nPCR were 35 cycles of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 90 s. The DNA obtained from the reference strains *S. neurona*, *N. caninum* (Nc-Liv), *H. hammondi*, and *T. gondii* (CTG) were used as positive controls of the nPCR reactions. All cycling reactions were carried out using Mastercycler EP Gradient (Eppendorf, USA), and the obtained products were visualised under an UV transilluminator after electrophoresis in 1.5% agarose gel stained with ethidium bromide. The positive sample products were submitted for Sanger sequencing after purification using Wizard SV Gel and PCR Clean-Up System (A9281 – Promega, USA) and quantification using Hoefer DNA Quantitation DQ 200 fluorometer (Hoefer, USA). Sequencing of purified PCR products was performed with 5 mM of the nPCR internal primers and 50 ng of PCR purified product using the BigDye Terminator v.3.1 Cycle Sequencing Kit according to 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, 5′ and 3′-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.

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% (91/1921) 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 (29 from brain and 21 from pectoral muscle/heart samples). The results of the BLAST search of each nPCR-18S rDNA sequence are summarised in Table 1.

The analysis of the partial 18S rDNA sequences revealed that ten of them presented 100% similarity with previously described Sarcocystidae parasites: *T. gondii* (n = 5), *N. caninum* (n = 1), *S. (Frenkelia) glareoli* (n = 1), *H. hammondi* (n = 1), *Cystoisospora canis* (n = 1), and *N. eptesici* (n = 1). The remaining 40 sequences were grouped according to their

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	98.7% *Toxoplasma gondii* (MN595284)
M494[a]	*Molossus molossus*	Insectivorous	São Paulo	
	Molossus rufus	Insectivorous		
M500	*Molossus molossus*	Insectivorous	São Paulo	
M820	*Molossus molossus*	Insectivorous	São Paulo	
M826	*Molossus molossus*	Insectivorous	São Paulo	
M865	*Glossophaga soricina*	Nectarivorous	São Paulo	
M1109[a]	*Molossus molossus*	Insectivorous	São Paulo	
	Molossus molossus	Insectivorous		
	Molossus molossus	Insectivorous		
B481	*Molossus molossus*	Insectivorous	São Paulo	
B482	*Molossus molossus*	Insectivorous	São Paulo	
M1130	*Molossus molossus*	Insectivorous	São Paulo	
B44	*Molossus molossus*	Insectivorous	São Paulo	
B1345	*Molossus molossus*	Insectivorous	Jundiaí	
B1349	*Molossus molossus*	Insectivorous	Jundiaí	
M48,B651	*Eumops glaucinus*	Insectivorous	Piracicaba	
B1466	*Eumops glaucinus*	Insectivorous	São José do Rio Preto	100% *T. gondii*
M60	*Molossus molossus*	Insectivorous	São José do Rio Preto	(MN595284)
B1052	*Glossophaga soricina*	Nectarivorous	São Paulo	
B774	*Molossus molossus*	Insectivorous	Piracicaba	98.2% *T. gondii* (MN595284)
M853	*Platyrrhinus lineatus*	Frugivorous	São Paulo	97% *Nephroisospora epitesici* (EU334134)
B265	*Artibeus planirostris*	Frugivorous	São José do Rio Preto	
B469	*Artibeus lituratus*	Frugivorous	São Paulo	
B688	*Artibeus lituratus*	Frugivorous	Jundiaí	
B955	*Artibeus lituratus*	Frugivorous	Guarulhos	
B1002	*Glossophaga soricina*	Nectarivorous	São Paulo	
B1398	*Artibeus lituratus*	Frugivorous	São José do Rio Preto	
B1682	ND		São Paulo	
B1700	*Artibeus lituratus*	Frugivorous	São Paulo	
B1707	*Artibeus lituratus*	Frugivorous	São José dos Campos	
B1740	*Phyllostomus discolor*	Frugivorous	Piracicaba	
M886	*Platyrrhinus lineatus*	Frugivorous	São Paulo	
B1379	*Cynomops planirostris*	Insectivorous	Sorocaba	
B1342	*Myotis nigricans*	Insectivorous	São Paulo	97.4% *N. epitesici* (EU334134)
M1121	*Histiotus velatus*	Insectivorous	São Paulo	
B789	*Eptesicus furinalis*	Insectivorous	Jundiaí	100% *N. epitesici* (EU334134)
M493[a]	*Molossus molossus*	Insectivorous	São Paulo	
	Nyctinomops macrotis	Insectivorous		
	Sturnira lilium	Frugivorous		
M496[a]	*Nyctinomops macrotis*	Insectivorous	São Paulo	97.4% *Neospora caninum* (AJ271354)
	Molossus molossus	Insectivorous		
	Molossus molossus	Insectivorous		
		Insectivorous		
		Insectivorous		
M828	*Nyctinomops macrotis*	Insectivorous	São Paulo	
B657	*Nyctinomops laticaudatus*	Insectivorous	São José do Rio Preto	97.8% *N. caninum* (AJ271354)
B829	*Nyctinomops laticaudatus*	Insectivorous	Piracicaba	
B999	*Molossus molossus*	Insectivorous	São Paulo	
M1148	*Glossophaga soricina*	Nectarivorous	São Paulo	100% *N. caninum* (AJ271354)
B935	*Artibeus lituratus*	Frugivorous	São Paulo	97% *Besnoitia besnoiti* (XR_003828658)
M437	*Artibeus lituratus*	Frugivorous	Itu	96.1% *B. besnoiti* (XR_003828658)
B332	*Glossophaga soricina*	Nectarivorous	São Paulo	100% *Hammondia hammondi* (AH008381)
M308	*Nyctinomops laticaudatus*	Insectivorous	São José do Rio Preto	100% *Sarcocystis* (*Frenkelia*) *glareoli* (AF009245)
M591	*Molossus molossus*	Insectivorous	São José do Rio Preto	100% *Cystoisospora canis* (KT184368)
M222,B468	*Tadarida brasiliensis*	Insectivorous	São Paulo	96.1% *Hyaloklossia lieberkuehni* (AF298623)
B159	*Nyctinomops laticaudatus*	Insectivorous	São José do Rio Preto	97.4% *Cystoisospora belli* (JX025652)

M: muscle; B: brain; ND: not determined; [a]: DNA extracted from more than one bat specimen (tissue pool).

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 glaucinus* insectivorous bat: ToxoDB-RFLP non-archetypal genotypes #6 (type BrI, Africa 1) and #69 (Table 2). An incomplete genotype was obtained from one *Molossus molossus* bat, with alleles II and I at GRA6 and Apico markers, respectively.

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).

Bat species		PCR-RFLP markers											Identity with other *T. gondii* samples	Host	ToxoDB* RFLP genotype
Sample[#]		SAG1	5′- 3′ SAG2	Alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	CS3			
Eumops glaucinus	M48	I	III	III	III	III	II	I	III	I	II	I	I	[1]TgCkBr21, 93, 94	Chicken	#69
Eumops glaucinus	B651 B1466	I	I	I	**III**	I	**II**	**u-1**	I	I	I	I	I	[2]TgCkBr4, 10, 21, 55, 79, 86, 87, 98, 101, 102, 104, 123, 124, 144, 201, 203, 207, 265, 273, 277, 281, Es4,5, MA4, CH4,5, ChBrUD2, Pr2,3; [3]TgCatBr2, 12, 17, 21, 30, 42, 47, 53, 54, 55, 62, 71, 75; [4]TgDgBr3, 7, 20; [5]TgShBr8, 9, 10, 11; [6]TgGtBr2, 3, 4, 9; [7]TgCpBr14; [8]TgSbaBr2; [9]RR09; [10]TgDoveBr7; [11]534N; [12]PL8, SIPF2; [13]WIK, PSP-KOM, TRK1, BOF, GPHT, FOU	Chicken, cat, dog, sheep, goat, capybara, banded-armadillo, black rat, eared dove, human	**#6 (Type BrI, Africa 1)**

u-1: atypical allele (Su et al., 2006); *Toxo DB: http://toxodb.org/toxo/; [#]M: muscle, B: brain.
[1,2]Isolates from chickens from Brazil (Dubey et al., 2020, review); [3]Isolates from cats from Brazil (Pena et al., 2006; Dubey et al., 2004); [4]Isolates from dogs from Brazil (Dubey et al., 2007); [5,6]Isolates from sheep and goats from Brazil, respectively (Ragozo et al., 2010); [7]Isolate from capybara from Brazil (Yai et al., 2008); [8]Isolate from *Euphractus sexcinctus* from Brazil (Vitaliano et al., 2014); [9]Isolate from *Rattus rattus* from Brazil (Aratijo et al., 2010); [10]Isolate from *Zenaida auriculata* from Brazil (Barros et al., 2014); [11]Human sample from Brazil (Ferreira et al., 2011); [12]Isolates from chickens from Gabon (Dubey et al., 2020, review); [13]Isolates from humans from Cameron, Turkey, Belgium, French Republic (Shwab et al., 2014).

4. 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 2013; Kirillova et al., 2018; Cesar et al., 2018).

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 cystoisoporinae, toxoplasmatinae, and sarcocystinae 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 insectivorous bats *M. molossus* and *Nyctinomops 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* insectivorous 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 insectivorous and nectarivorous 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 (Shwab 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 (Shwab 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* insectivorous 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 BrI or Africa 1) is widely distributed across Africa and Brazil and in different host animals, including humans (Shwab et al., 2014). However, it has been identified to circulate in Chiroptera for the first

time. These results corroborate the high genetic variability of *T. gondii* in Brazil and the importance of bats as natural intermediate host for this zoonotic protozoan. Furthermore, as 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

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Declaration of interests

None.

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