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RESEARCH

Phylogenetic analysis of a novel *Hepatozoon* species (*Hepatozoon* sp. SK3) and an additional yet unknown *Hepatozoon* species (*Hepatozoon* sp. BV2) besides *H. erhardovae* in small rodents from Central Europe

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Abstract

Hepatozoon spp. are tick-borne apicomplexan parasites of terrestrial vertebrates that occur worldwide. Tissue samples from small rodents and their parasitizing fleas were sampled for molecular detection and phylogenetic analysis of *Hepatozoon*-specific 18S rRNA gene region. After alignment and tree inference the *Hepatozoon*-sequences retrieved from a yellow-necked mouse (*Apodemus flavicollis*) placed into a strongly supported single clade demonstrating the presence of a novel species, designated *Hepatozoon* sp. SK3. The mode of transmission of *Hepatozoon* sp. SK3 is yet unknown. It is important to note that this isolate may be identical with the previously morphologically described *Hepatozoon sylvatici* infecting *Apodemus* spp.; however, no sequences are available for comparison. Furthermore, the previously reported variants *Hepatozoon* sp. BV1/SK1 and BV2/SK2 were detected in bank voles (*Clethrionomys glareolus*). It has been suggested that these variants should be identified as *Hepatozoon erhardovae* leading to the assumption that BV1 and BV2 are paralogous 18S rRNA gene loci of this species. Evidence has also been presented that fleas are vectors of *H. erhardovae*. In this study, we show with high significance that only the *Hepatozoon* sp. BV1 variant, but not BV2, infects the studied flea species *Ctenophthalmus agyrtes*, *Ctenophthalmus assimilis*, and *Megabothris turbidus* ($p < 0.001$). This finding suggests that *Hepatozoon* sp. BV2 represents an additional species besides *H. erhardovae* (=*Hepatozoon* sp. BV1), for which alternative arthropod vectors or non-vectorial modes of transmission remain to be identified. Future studies using alternative molecular markers or genome sequencing are required to demonstrate that BV1/SK1 and BV2/SK2 are different *Hepatozoon* species.

Keywords *Hepatozoon* · Small rodents · *Apodemus flavicollis* · *Clethrionomys glareolus* · Fleas · Central Europe

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Introduction

The apicomplexan genus *Hepatozoon* comprises intracellular blood parasites that have been described from a wide range of terrestrial vertebrates such as amphibians, reptiles, and mammals (Smith 1996; Maia et al. 2014, 2016; Baneth and Allen 2022). *Hepatozoon* exhibits an obligate heteroxenous life cycle in which bloodsucking arthropods, such as fleas, ticks, mosquitoes, mesostigmatid mites, and sandflies acquire the infection by feeding on parasite-infected blood cells of vertebrates (Rigó et al. 2016). In turn, intermediate vertebrate hosts become infected through the ingestion of *Hepatozoon*-infected arthropods, which serve as vector and definitive host (Smith 1996). After ingestion, the parasite enters blood circulation and migrates to various organs including liver and spleen.

Hepatozoon infection of wildlife animals, such as small rodents, is considered asymptomatic. In contrast, domestic dogs present clinical disease and the most common symptoms of the infection of pathogenic *H. canis* or *H. americanum* include anemia, lethargy, weight loss, weakness, and cachexia. Canine hepatozoonosis is transmitted either by ingestion of *H. canis*-infected ticks (*Rhipicephalus sanguineus*) or *H. americanum*-infected ticks (*Amblyomma maculatum*). However, transplacental transmission has also been reported (Baneth and Allen 2022).

Hepatozoon spp. have been identified in wild small rodents from many regions in Europe (Laakkonen et al. 2001; Criado-Fornelio et al. 2006; Bajer et al. 2001, 2014; Hamšíková et al. 2016; Rigó et al. 2016; Galfsky et al. 2019; Modrý et al. 2021; Ferrari et al. 2022; Uiterwijk et al. 2023). At least three extensive molecular epidemiological studies, in which rodent associated *Hepatozoon* spp. could not be detected in ticks suggest that other arthropod species or non-vectorial routes maintain the transmission cycle of these hemoprotozoans in rodents (Hamšíková et al. 2016; Rigó et al. 2016; Uiterwijk et al. 2023).

Accordingly, Rigó et al. (2016) and Špitalská et al. (2022) detected *Hepatozoon*-positive fleas collected from small rodents in Hungary and Slovakia, respectively. Both studies found that the 18S rRNA gene fragments recovered from fleas and an unrecognized isolate designated *Hepatozoon* sp. SK3 were too short for in-depth phylogenetic analysis (Hamšíková et al. 2016). In the present study, we recovered extended 18S rRNA gene sequences from these and additional tissue samples of small rodents and fleas allowing us to carry out a phylogenetic analysis to delineate and reveal the identity of these *Hepatozoon* isolates.

Materials and methods

DNA extraction, PCR amplification, and sequence analysis

Rodents ($n = 10$) were previously sampled in southwestern Slovakia (Hamšíková et al. 2016) and southern Hungary (Rigó et al. 2016). DNA was extracted from rodent spleen (n = 10) and lungs (n = 5) using a commercial isolation kit. Fleas ($n = 6$) found on trapped animals were stored in 70% ethanol until genomic DNA was isolated by alkaline hydrolysis (Rigó et al. 2016). Rodent and flea genomic DNA samples were screened by PCR targeting a *Hepatozoon*-specific 635 bp fragment of the 18S rRNA gene using specific primers as previously published (Ujvari et al. 2004). PCR products were sequenced in both directions using amplification primers (Macrogen, South-Korea). Nucleotide sequences were assembled using Bioedit and used as queries for nBLAST search in GenBank to find closest hits. Sequences were deposited in GenBank under Accession numbers (PP420928-PP420948; Table S1, supplementary material).

Phylogenetic analysis

Molecular phylogenetic analysis was conducted on a dataset of analyzed and closely related relevant 18S rRNA gene sequences available in GenBank as to February 2024. Altogether 51 sequences were aligned using Muscle. A maximum likelihood tree was inferred based on the T92 (+ G) model using a Gamma distribution (G = 0.29) with a rate difference of 5 categories. For tree estimation, 572 positions were used. As outgroup the 18S rRNA gene sequence of *Adelina bambarooniae* (AF494059) was used. Branch support was estimated by running 1000 nonparametric bootstraps. Phylogenetic analysis was carried out using MEGA11 (Tamura et al. 2021).

Results and discussion

Hepatozoon infections of small rodents are frequent and depending on the geographic region of Europe, a prevalence of up to 64.2% *H. erhardovae* in bank voles (*C. glareolus*) and of up to 32.2% *Hepatozoon* spp. in yellow-necked mice (*A. flavicollis*) has been reported (Laakkonen et al. 2001; Bajer et al. 2014; Rigó et al. 2016; Hamšíková et al. 2016; Ferrari et al. 2022; Uiterwijk et al. 2023).

Fifteen high-quality *Hepatozoon* spp. 18S rRNA gene sequences of 549 to 585 bp-length were obtained from an individual yellow-necked mouse (*A. flavicollis*; spleen

$n = 1$, lung $n = 1$) and from nine bank voles (*C. glareolus;* spleen $n = 9$, lung $n = 4$) (Table S1, supplementary material). The two 18S rRNA gene sequences recovered from the yellow-necked mouse showed a 100% identity with the previously reported partially overlapping sequences of *Hepatozoon* sp. SK3 (KU597250-51, 502 nt) (Hamšíková et al. 2016). Importantly, the second best hit to this sequence (KX453636, *Hepatozoon* sp. from a crowned leaf nose snake) showed only an identity of 92.32% strongly suggesting that this isolate represents a novel yet unrecognized species. Phylogenetic analysis confirmed this assumption since both 18S rRNA gene sequences placed into a single strongly supported clade (bootstrap: 91) (Fig. 1). It is currently unclear whether *Hepatozoon* sp. SK3 is the same as *H. sylvatici,* a species previously described morphologically in *Apodemus* spp., since there is currently no sequence available for this parasite (Frank 1977; Walter and Liebisch 1980). In future studies, 18S rRNA gene sequence analysis of both *Hepatozoon* sp. SK3 and *H. sylvatici* is necessary to prove or disprove their identity (as done for *H. erhardovae,* Rigó et al. 2016). It is interesting to note that Ferrari et al. (2022) reported the sequence of the 18S rRNA gene TN-3, which is identical to that of *Hepatozoon* sp. SK3, but did likewise not recognize this isolate as a new species.

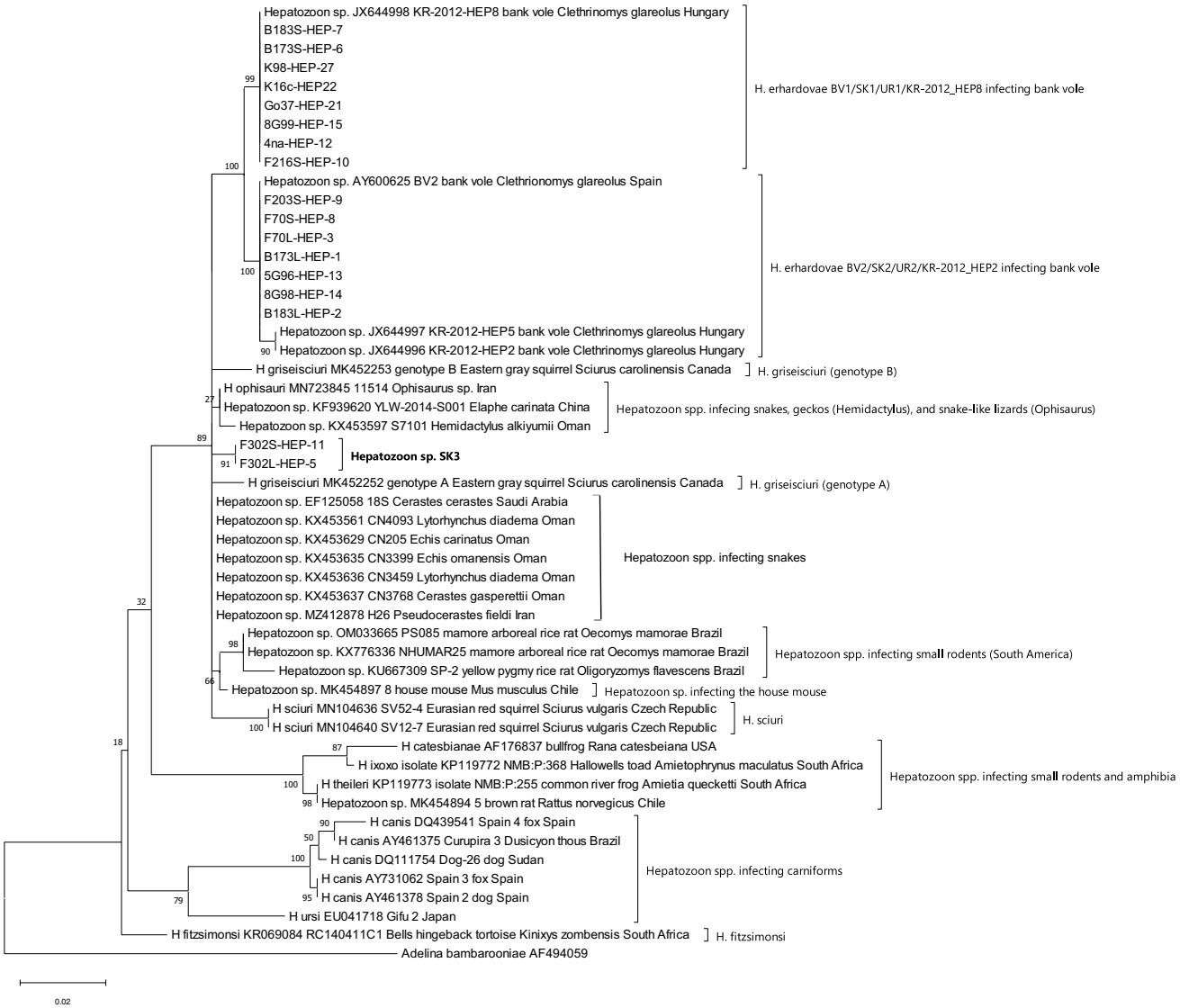

Fig. 1 Maximum likelihood phylogenetic tree including 18S rRNA gene sequences of *Hepatozoon* spp. infecting rodents and fleas. The length of the bar corresponds with the indicated number of substitutions per site. Bootstrap values are given at tree nodes. The evolutionary distance is shown in the units of the number of base substitutions per site. The name that identifies the clade of the novel species *Hepatozoon* sp. SK3 is shown in bold. Sequences are marked by their species name, accession numbers, isolate designation, host vertebrate or host vector, and country of origin as retrieved from GenBank. Sequences analyzed in this study are indicated only with their isolate designation (see Table S1, supplementary material)

In addition, the recent misidentification of *Hepatozoon* sp. SK3 in a recent report is noteworthy, and resulted in the misnaming of GenBank entries KT27477-78 as *H. ayorgbor* (Uiterwijk et al. 2023). Notwithstanding, the identification of an 18S rRNA gene sequence identical to that of the isolate *Hepatozoon* sp. SK3 (KU597250-51) in *Apodemus sylvaticus* (KT27477) and *A. flavicollis* (KT27478) by Uiterwijk et al. (2023) strongly suggests that this *Hepatozoon* species infects both of these rodent species.

Thirteen of the fifteen *Hepatozoon* spp. 18S rRNA sequences of 549 to 585 bp-length were obtained from the spleen and/or lung of nine individual bank voles (*C. glareolus*; spleen $n=9$, lung $n=4$) (Table S1, supplementary material). From three of the nine bank vole specimens, B173, F70, and F216, the sequences KU597252, KU597253, and KU597254 have been previously reported, respectively (Table S1, supplementary material; Hamšíková et al. 2016). In the present study, a different partially overlapping 18S rRNA gene region, more suitable for a phylogenetic analysis, was recovered from all nine *C. glareolus* specimens.

In all cases, the 18S rRNA gene sequence amplicons obtained from the nine *C. glareolus* specimens showed 100% identity either with the sequence of *Hepatozoon* sp. BV1 (AY600626) or with that of *Hepatozoon* sp. BV2 (AY600625). In two of the nine *C. glareolus* specimens (B173, B183), the sequences BV2 and BV1 were identified from lung and spleen, respectively. From lung and spleen of another two *C. glareolus* samples, the sequence pair BV2/BV2 (specimen F70) and BV1/BV1 (specimen F216) was recovered. Finally, in the spleen of the remaining five *C. glareolus* rodents, the sequence BV1 could be identified in two (4n.a., 8G99), and the sequence BV2 in three specimens (F203, 5G96, 6G98).

It is noteworthy that the two sequence variants AY600626 and AY600625 were first reported as *Hepatozoon* spp. BV1 and BV2 isolates from bank voles in Spain, and it was suggested that they may represent *H. erhardovae* (Criado-Fornelio et al. 2006). Rigó et al. (2016) were able to confirm this notion by showing that the sequences *Hepatozoon* sp. KR-2012_HEP2 (JX644996 similar to AY600625, *Hepatozoon* sp. BV2) and *Hepatozoon* sp. KR-2012_HEP8 (JX644998 similar to AY600626, *Hepatozoon* sp. BV1) were found to be associated with the occurrence of *H. erhardovae* gamonts in bank voles. In additional studies, the corresponding two 18S rRNA gene variants have been recovered and those similar to *Hepatozoon* sp. BV1 (AY600626) were referred to as *Hepatozoon* sp. SK1 (KU597252 and KU597254) or *H. erhardovae* UR1_2010 (KF418366). On the other hand, those identical to *Hepatozoon* sp. BV2 (AY600625) were referred to as *Hepatozoon* sp. SK2 (KU597253) or *H. erhardovae* UR2_2010 (KF418367) (Bajer et al. 2014; Hamšíková et al. 2016). Furthermore, *Hepatozoon* spp. have also been reported in bank voles from Germany (Galfsky et al. 2019). However, recovered sequences were short (177 to 236 bp) and a robust species determination could not be carried out. The analysis of longer sequences will be required to unequivocally confirm the presence of *Hepatozoon* sp. BV1 and BV2 in this country. Thus, the variant BV1 has been also designated SK1, UR1, or KR-2012_HEP8, while BV2 was referred to as SK2, UR2, or KR-2012_HEP2 (Criado-Fornelio et al. 2006; Bajer et al. 2014; Rigó et al. 2016; Hamšíková et al. 2016). Importantly, both genetic variants seem to coexist in bank voles in studied geographic areas of Europe including Spain, Slovakia, Czech Republic, Poland, and Hungary (Bajer et al. 2014; Rigó et al. 2016).

In the present study, a phylogenetic analysis was carried out based on an alignment of the *Hepatozoon* 18S rRNA gene sequences analyzed and other closely related sequences available in GenBank. In the inferred tree the sequences recovered from bank voles segregated either into the well supported branch comprising *Hepatozoon* sp. BV1 and KR2012_HEP8 (bootstrap: 99) or into the branch including *Hepatozoon* sp. BV2 and KR2012_HEP5 (bootstrap: 100). Thus, phylogenetic analysis allows to clearly distinguish both sequence variants by their placement into two different strongly supported sister clades (Fig. 1, Hamšíková et al. 2016).

It had been proposed that the two sequence variants represent paralogous 18S rRNA gene loci encoded in the genome of *H. erhardovae* (Fig. 1, Hamšíková et al. 2016). Correspondingly, independent amplification and sequence analysis of each variant from single rodent samples, but also the occurrence of corresponding double peaks in at least three rodent specimens (data not shown) was observed. However, polymorphism corresponding to paralogous 18S rRNA gene sequences has not been observed in any of the other molecularly analyzed *Hepatozoon* spp. and is known to be an extremely rare observation in other protozoan species. Alternatively, the finding of two different 18S rRNA genes may be due to the presence of an additional yet unrecognized *Hepatozoon* species (*Hepatozoon* sp. BV2) co-infecting bank voles with *H. erhardovae* (*Hepatozoon* sp. BV1). In future studies, this could be analyzed using additional genetic markers, such as the *cox-1* (cytochrome c oxidase I) gene, or by genome sequencing. The advantage of the *cox-1* gene for species delineation in *Hepatozoon* and other Apicomplexan parasites such as Piroplasmida has been outlined by Schnittger et al. (2012, 2022) and Thomas et al. (2024).

Using standard identification keys, *Hepatozoon*-positive flea species *Ctenophthalmus agyrtes* ($n=5$) and *Megabothris turbidus* ($n=1$) were identified as previously described (Rigó et al. 2016). Sequences of 420 bp (KJ608372) and 427 bp (KJ634066) were recovered from the flea isolates 44a and K126b, respectively, but found to be too short for phylogenetic analysis. In the present study, these isolates

were therefore re-sequenced and an extended 18S rRNA gene fragment could be obtained from the six flea samples (Table S1, supplementary material; Rigó et al. 2016). All recovered sequences (549 or 585 nt) were found to be identical to each other and showed a 100% identity with the BV1 isolate from Spain (*Hepatozoon* sp. BV1, AY600626; Criado-Fornelio et al. 2006), corresponding to the 18S rRNA gene variant *Hepatozoon* sp. SK1 (KU597252 and KU597254), *H. erhardovae* sp. UR1 (KF418366), and *Hepatozoon* sp. KR-2012_HEP8 (JX644998).

Remarkably, in contrast to rodents, only the *Hepatozoon* sp. BV1 variant, but not BV2, was recovered from all six flea samples. This finding allows to reject the assumption that BV1 and BV2 represent paralogous genetic variants of two 18S rRNA gene loci from a single *H. erdhovanae* genome, but also that fleas are co-infected with two different *Hepatozoon* species or variants (binominal probability P(x=6:n=6,p=0.5) => $p < 0.05$. Importantly, a recent study confirmed our finding with high significance since only the *Hepatozoon* sp. BV1 (=SK1) variant was detected in a total of fourteen *Hepatozoon*-positive fleas of the species *Ctenophthalmus assimilis* ($n=1$), *C. agyrtes* ($n=12$), and *M. turbidus* ($n=1$) (binominal probability P(x=14:n=14,p=0.5) => $p \ll 0.001$; Špitalská et al. 2022). The biological significance of this observation remains to be determined, but the hypotheses that BV1 and BV2 represent paralogous gene loci of a single *Hepatozoon* species, or that two *Hepatozoon* sp. BV1 and BV2 infect fleas must be rejected. At present, data are best reconciled by assuming the presence of two different species of which only *Hepatozoon* sp. BV1 is transmitted by fleas, while *Hepatozoon* sp. BV2 is likely transmitted by an alternative, yet unrecognized vector or by a non-vectorial route. As a non-vectorial transmission route carnivorism between mammalians either by hunting or by scavenging has been reported (Baneth et al. 2013; Thomas et al. 2024). Again, as mentioned above, the use of additional genetic markers or genome sequencing would allow to provide supporting evidence whether *Hepatozoon* spp. BV1 and BV2 represent distinct parasite species or populations.

Although *Hepatozoon* spp. are often considered to have low host specificity, our phylogenetic tree suggests that *Hepatozoon* spp. show a pronounced host specificity as cross-infection between different host groups or species is rarely observed (Fig. 1). These rare reports of cross-infections seem to be rather spill-over effects or are due to misidentification of *Hepatozoon* spp. of which a number of examples are given above. Thus, *Hepatozoon* spp. infecting small rodents seem not to infect snakes, snake-like lizards (*Ophisaurus* sp.), or geckos and vice versa. Furthermore, *Hepatozoon* sp. BV1/BV2 and *Hepatozoon* sp. SK3 display host specificity for bank voles (*C. glareolus*) and wild mice (*Apodemus* spp.), respectively. In addition, monophyletic groups of *Hepatozoon* spp. have been reported that either infect exclusively amphibia/rodents, South American rodents, or squirrels (Modrý et al. 2021). Finally, *Hepatozoon* spp. infecting caniforms are principally not detected in rodents and vice versa.

Importantly, a number of extensive molecular epidemiological studies could not detect rodent-associated *Hepatozoon* spp. in ticks, strongly suggesting the implication of other arthropod vector species or non-vectorial routes in the transmission cycle of hemoprotozoans in rodents (Hamšíková et al. 2016; Špitalská et al. 2022; Uiterwijk et al. 2023). Špitalská et al. (2022) and the present study provide evidence that fleas are candidate vectors for *Hepatozoon* sp. BV1, since only this variant, but not BV2 could be detected in fleas. Finally, our data allow to discard that BV1 and BV2 are paralogous 18S rRNA genes, strongly suggesting that each represents a molecular marker for a *Hepatozoon* species. Molecular studies are needed to present direct evidence that *Hepatozoon* sp. BV1 and BV2 are distinct species.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00436-024-08269-z.

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Author contribution Conceptualization: LS, MK, and GF; methodology: LS and SG; formal analysis: SG and LS; resources: GF, SS, ZH, MK, and LS; data curation: SG; investigation: SG and LS; writing—original draft: SG and LS; writing—review and editing: LS, SG, GF, SS, MP, MK, and LS; visualization: LS and SG; supervision: LS; funding acquisition: LS, SG, FG, and MK. All authors have read and approved the content of the submitted manuscript.

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Declarations

Competing interests The authors declare no competing interests.

Ethical approval Rodent trapping and handling in Slovakia were approved by the Regional Environmental Office in Bratislava (licence ZPO-594/2012-SAB) and comply with current laws of the Slovak Republic. Sample collection was carried out with official permission from the Middle Transdanubian Inspectorate for Environmental Protection, Natural Protection and Water Management, Hungary.

Consent to participate Not applicable.

Consent for publication We declare that all authors have read and approved the manuscript for submission to Parasitology Research.

Conflict of interest The authors declare no competing interests.

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