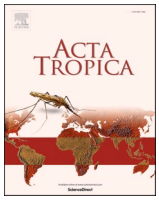
Acta Tropica 252 (2024) 107136

Contents lists available at ScienceDirect

Acta Tropica

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



First molecular detection and characterization of *Enterocytozoon bieneusi* different genotypes in human patients from Italy

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ARTICLE INFO

Keywords:
Microsporidia
Enterocytozoon bieneusi
Gastrointestinal parasites
Genotypes A, C, K

ABSTRACT

Enterocytozoon bieneusi is one of the 17 microsporidian species pathogenic to humans in low and high-income countries, inducing both symptomatic and asymptomatic intestinal infections, independently of the immunological condition of the infected individual. Faecal-oral transmission occurs in a broad hosts range, including several animal species, but the parasite's zoonotic potential remains still unclear. Few studies are available in Italy regarding *E. bieneusi* presence in humans and no data on its genetic variability are so far reported. In this investigation, through the ITSr RNA sequences analysis, we provided the first *E. bieneusi* molecular characterization from symptomatic patients in Italy. Faecal samples from 410 patients sent for routine analyses to the Unit of Parasitology, Policlinico Tor Vergata, Rome, and resulted positive for *E. bieneusi* to a cartridge-based molecular test for qualitative detection (Novodiag® Stool Parasites assay), were collected. DNA was extracted, endpoint PCR performed and then sequences obtained for 3/410 patients (0.7 %). Genotype A ($N = 1$), genotype C ($N = 1$) and genotype K ($N = 1$) were identified, all belonging to phylogenetic Group 1. One patient (identified as genotype A) showed positivity to the same genotype previously characterized after a two-month period. Additional investigations are required, within a One Health framework, to review the importance of a zoonotic potential linked to *E. bieneusi* in human populations, animals and environmental reservoirs worldwide.

1. Introduction

Microsporidia are a diversified group of fungal-related intracellular parasites considered as opportunistic pathogens. They encompass approximately 220 genera and 1.700 species, and their classification is primarily determined by their ultrastructural characteristics, developmental cycle, host-parasite interactions, and molecular analysis (Han et al., 2021).

Vertebrate such as invertebrate hosts can harbour Microsporidia. Human infections can exhibit a broad spectrum of manifestations, encompassing temporary gastrointestinal complications in immunocompetent patients, all the way to severe and potentially fatal conditions observed in individuals with compromised immune systems, including transplant patients, children, and the elderly (Akinbo et al., 2012; Dumond et al., 2021; Rezaeian et al., 2023). The majority of human microsporidiosis cases reported worldwide are caused by the genus *Encephalitozoon*, and by *Enterocytozoon bieneusi*, the only member of the genus *Enterocytozoon* pathogenic to humans, which infection mainly causes chronic diarrhoea and biliary disease (Han et al., 2021). *Enterocytozoon bieneusi* was initially identified in 1985 as a pathogen responsible for gastrointestinal infection characterized by persistent diarrhoea in individuals with advanced HIV-1 infections (Desportes et al., 1985). The parasite lifecycle occurs through the ingestion of spores that are resistant to the environment, with a faecal-oral transmission (Li et al., 2019a) Indeed, *E. bieneusi* infections in humans may occur by zoonotic transmission, by consumption of spores from environmental sources, as well as due to the human-to-human transmission (Li and Xiao, 2020).

Enterocytozoon bieneusi has also been identified in several animal hosts, ranging from dogs, pigs, raccoons, wild boar, and pigeons to waterfowl (Sulaiman et al., 2003b; Mathis et al., 2005; Slodkowicz-Kowalska et al., 2006), as well in water bodies (Mathis et al., 2005), raising a concern for zoonotic transmission. Despite this, it is still unclear how animals contribute to the spread of this parasite to humans, and its importance for public health remains debatable.

The treatment of microsporidiosis is still a challenge, and current therapies do not completely eradicate the pathogen. It is also known that *E. bieneusi* does not respond correctly to the drug of choice available,

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https://doi.org/10.1016/j.actatropica.2024.107136
Received 23 November 2023; Received in revised form 25 January 2024; Accepted 28 January 2024
Available online 29 January 2024

albendazole (Conteas et al., 2000). Therefore, preventing its transmission between susceptible hosts could reduce the infection risk. This is possible through genotyping tools able to identify the source of infection and the epidemiological patterns (Li et al., 2019b). End-point PCR and commercial real-time PCR (RT-PCR) panels have recently taken the place of microscopy in diagnostics. Since therapy is species-dependent, molecular methods provide a more accurate diagnosis and a quick identification of species and genotypes (Moniot et al., 2022).

Enterocytozoon bieneusi molecular characterization is based on the sequencing of the internal transcribed spacer (ITS) of rRNA. This region has exhibited a significant level of diversity among isolates, leading to a partially overlapped nomenclature, with certain genotypes assigned to multiple names depending on the author (Santín and Fayer, 2009). At least 685 different *E. bieneusi* genotypes from 13 different genetic groups have been described so far, with 106 harboured by humans (Jiang et al., 2023). The different groups exhibit diverse levels of host specificity and zoonotic potential, with Group 1 representing the most extensive cluster, encompassing more than 300 distinct genotypes that have been isolated from nearly all known host types. Notably, certain genotypes within this group, such as D, EbpC, and Type IV, have demonstrated an exceptional adaptability to various host and natural environments (Li et al., 2019b).

With regard to Italy, very few outdated studies have been conducted, all microscopy based, related to the presence of this parasite, exclusively with HIV patients as a target (Caramello et al., 1995; Boldorini et al., 1996; Voglino et al., 1996; Maggi et al., 2000; Dionisio et al., 1998).

In this work we present the first data regarding the detection and the molecular characterization of *E. bieneusi* in human patients from an Italian public hospital in Rome, Italy, with the identification of three different genotypes (A, C and K).

2. Materials and methods

The study was conducted in the Unit of Parasitology of the Azienda Ospedaliera Universitaria Policlinico Tor Vergata (PTV) of Rome, Central Italy, which handles about 48.000 accesses annually. In the period from November 2022 to July 2023, 499 faecal samples from 410 patients sent for analysis to the Unit of Parasitology were routinely tested for gastrointestinal pathogens through the commercial panel Novodiag® Stool Parasites assay (Mobidiag, Espoo, Finland), a cartridge-based molecular test for qualitative detection of protozoa, helminths, and microsporidia, according to the manufacturer's instructions. Its microarray technology allows for multiplex testing. Screening included all patients which faecal samples enrolled for a suspected parasitic infection in the gastrointestinal tract. These were symptomatic individuals for which a parasitological examination of the stool had been required by the physician. Demographic data (age, sex) were collected.

Diagnostic laboratory results and patient data were combined and anonymized before being processed. All procedures performed in this study were in accordance with the 1964 Helsinki Declaration and its

Table 1
E. bieneusi reference sequences dataset used for molecular identification and phylogenetic analysis. Genotypes are divided by phylogenetic group. Information on genotype, host, isolate, country of origin and Genbank Acc. ID are presented.

GROUP	Acc. Number	Genotype	Isolate	Host	Country	Reference
GROUP 1	OR676939	genotype A	EbITA_1	*H. sapiens*	Italy	**Present study**
GROUP 1	OR676940	genotype A	EbITA_1RI	*H. sapiens*	Italy	**Present study**
GROUP 1	OR676941	genotype K	EbITA_2	*H. sapiens*	Italy	**Present study**
GROUP 1	OR676942	genotype C	EbITA_3	*H. sapiens*	Italy	**Present study**
GROUP 1	DQ683746	CAF1	G0084	*H. sapiens*	Gabon and Cameroon	Breton et al. (2007)
GROUP 1	GU198951	CZ3	2288	*H. sapiens*	Czech Republic	Sak et al. (2011)
GROUP 1	AF076040	EbpA	–	*S. scrofa*	Switzerland	Breitenmoser et al. (1999)
GROUP 1	AF101197	genotype A	–	*H. sapiens*	Germany and Switzerland	Rinder et al. (1997)
GROUP 1	AY357195	genotype A	clone 15	*H. sapiens*	Bangkok	Leelayoova et al. (2005)
GROUP 1	MN136776	genotype A	561	*H. sapiens*	China	Qi et al. (2020)
GROUP 1	OQ091359	genotype A	11VIH	*H. sapiens*	Spain	Chozas et al. (2023)
GROUP 1	AF101199	genotype C	–	*H. sapiens*	–	Rinder et al. (1997)
GROUP 1	MK256484	genotype C	L10	*V. vulpes*	Poland	Perec-Matysiak et al. (2021)
GROUP 1	AF267141	genotype K	–	*F. catus*	–	Dengjel et al. (2001)
GROUP 1	MF693832	genotype K syn. 004–52	OS1917	*R. unicolor*	Australia	Zhang et al. (2018)
GROUP 1	JN997477	Nig1	25,121-Nig1	*H. sapiens*	Nigeria	Akinbo et al. (2012)
GROUP 1	AY371281	Peru6	7939	*H. sapiens*	Perù	Sulaiman et al. (2003a)
GROUP 1	FJ439677	S1	–	*H. sapiens*	Malawi	ten Hove et al. (2009)
GROUP 1	FJ439682	S6	–	*H. sapiens*	Malawi	ten Hove et al. (2009)
GROUP 1	OP851356	TypeIV	RMAL5	*V. vulpes*	Portugal	Figueiredo et al. (2023)
GROUP 1	AY237215	WL7	3599	Castor	Maryland	Sulaiman et al. (2003b)
GROUP 1	AY237218	WL10	5489	*O. zibethicus*	Maryland	Sulaiman et al. (2003b)
GROUP 1	UG2145	UG2145	UG2145	*H. sapiens*	Uganda	Tumwine et al. (2002)
GROUP 2	MK982507	BEB4	PC53	Calf	Bangladesh	Karim et al. (2022)
GROUP 2	AF135836	genotype1	–	Cattle	–	Rinder et al. (2000)
GROUP 3	AY237212	WL4	3666	*O. zibethicus*	Maryland	Sulaiman et al. (2003b)
GROUP 3	AY237214	WL6	5540	*O. zibethicus*	Maryland	Sulaiman et al. (2003b)
GROUP 3	DQ885584	PtEb VIII	PtEb VIII	*F. s. catus*	Portugal	Lobo et al. (2006)
GROUP 4	AY237209	WL1	5496	*P. lotor*	Maryland	Sulaiman et al. (2003b)
GROUP 4	AY237210	WL2	3608	*P. lotor*	Maryland	Sulaiman et al. (2003b)
GROUP 4	JQ863275	WW7	19,107	Wastewater	China	Li et al. (2012)
GROUP 5	DQ683749	CAF4	G0475	*H. sapiens*	Gabon	Breton et al. (2007)
GROUP 5	JF681180	KB-6	27,920	*P. papio*	Kenya	Li et al. (2011)
GROUP 6	GQ406054	horse 2	–	*E. ferus*	Colombia	Santín et al. (2010)
GROUP 7	KJ668732	CD5	ZD-380	*C. lupus familiaris*	China	Karim et al. (2014a)
GROUP 7	KF543866	CM4	S-147	*R. roxellana*	China	Karim et al. (2014b)
GROUP 8	KJ867480	DeerEb1	4	*O. virginianus*	USA	Santín and Fayer (2015)
GROUP 8	KJ867485	DeerEb6	52	*O. virginianus*	USA	Santín and Fayer (2015)
GROUP 9	KR062125	WR7	28-M-11S	*A. agrarius*	Poland	Perec-Matysiak et al. (2015)
GROUP 9	KR062123	WR9	8-S-11F	*A. flavicollis*	Poland	Perec-Matysiak et al. (2015)
GROUP 10	FJ439683	S7	–	*H. sapiens*	Netherlands	ten Hove et al. (2009)
OUTGROUP (GROUP 11)	AF059610	PtEb IX	–	–	–	Mathis et al. (1999)

later amendments.

Once obtained the positivity to *E. bieneusi*, the stool samples were collected, kept at 4 °C, and within 24 h the genomic DNA extraction performed by a commercial kit (QIAamp DNA Stool Mini Kit, QIAGEN, Valencia, CA, USA). Molecular characterization was performed amplifying the ITS region and parts of the rRNA gene by a nested protocol as previously described (Buckholt et al., 2002), obtaining a 390 bp product. To ensure the PCR accuracy and reliability, both negative and positive controls were incorporated into each PCR run. Amplicons were purified through the mi-PCR Purification Kit (Metabion International AG) and then sent to an independent laboratory for sequencing (Bio-Fab Research, Rome, Italy). Forward and reverse sequences were manually checked using FinchTV. The obtained consensus sequences were then compared with those available in GenBank database by using the Standard Nucleotide BLAST search and aligned through AliView (Larsson, 2014), with representative sequences used as reference (Table 1). To enhance the evaluation of genetic diversity among *E. bieneusi* genotypes in this study and to establish genetic relationships between newly identified genotypes and previously described, a Maximum Likelihood (ML) phylogenetic tree was generated from the Table 1 dataset using the IQ-TREE software (von Haeseler et al., 2014). The construction of the phylogenetic tree was conducted utilizing the best model Hasegawa-Kishino-Yano (HKY) (Hasegawa et al., 1985) and represented in FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). To ensure the reliability of the genetic distances, a bootstrap analysis was performed with 1.000 replicates.

3. Results

A total of 410 patients has been evaluated and tested for suspected gastrointestinal symptoms, aged from 1 to 93 years old (average age 45 years; median age 47 years; sex ratio: 47.3 % males, 52.7 % females). Four faecal samples out of 499, all from diarrhoeal patients, resulted as positive to the cartridge-based molecular test for qualitative detection of *E. bieneusi*. The four isolates derived from three different individuals. Indeed, the first patient came back for a follow-up two months after his first positivity.

They were then successfully processed to the endpoint PCR amplification and subsequently sequenced. The obtained sequences resulted in a 390 bp fragment spanned the position 31–420 of the reference sequence Acc. ID AF101197. Analysis of the ITS rRNA region assigned them as follow:

- the identical sequences EbITA_1 and EbITA_1RI (where isolate EbITA_1RI was retrieved during the follow-up of the same patient source for EbITA_1) were assigned to genotype A (syn. genotype Peru1), showing 100 % of identity with the reference sequence AF101197 (query cover 100 %);
- isolate EbITA_2 was identified as genotype K (syn. genotype 004–52) differing from isolate EbITA_1 and EbITA_1RI for one single nucleotide polymorphism (SNP) (nucleotide transition C>T in position 243) and having 100 % of identity with sequence MF693832 (100 % query cover);

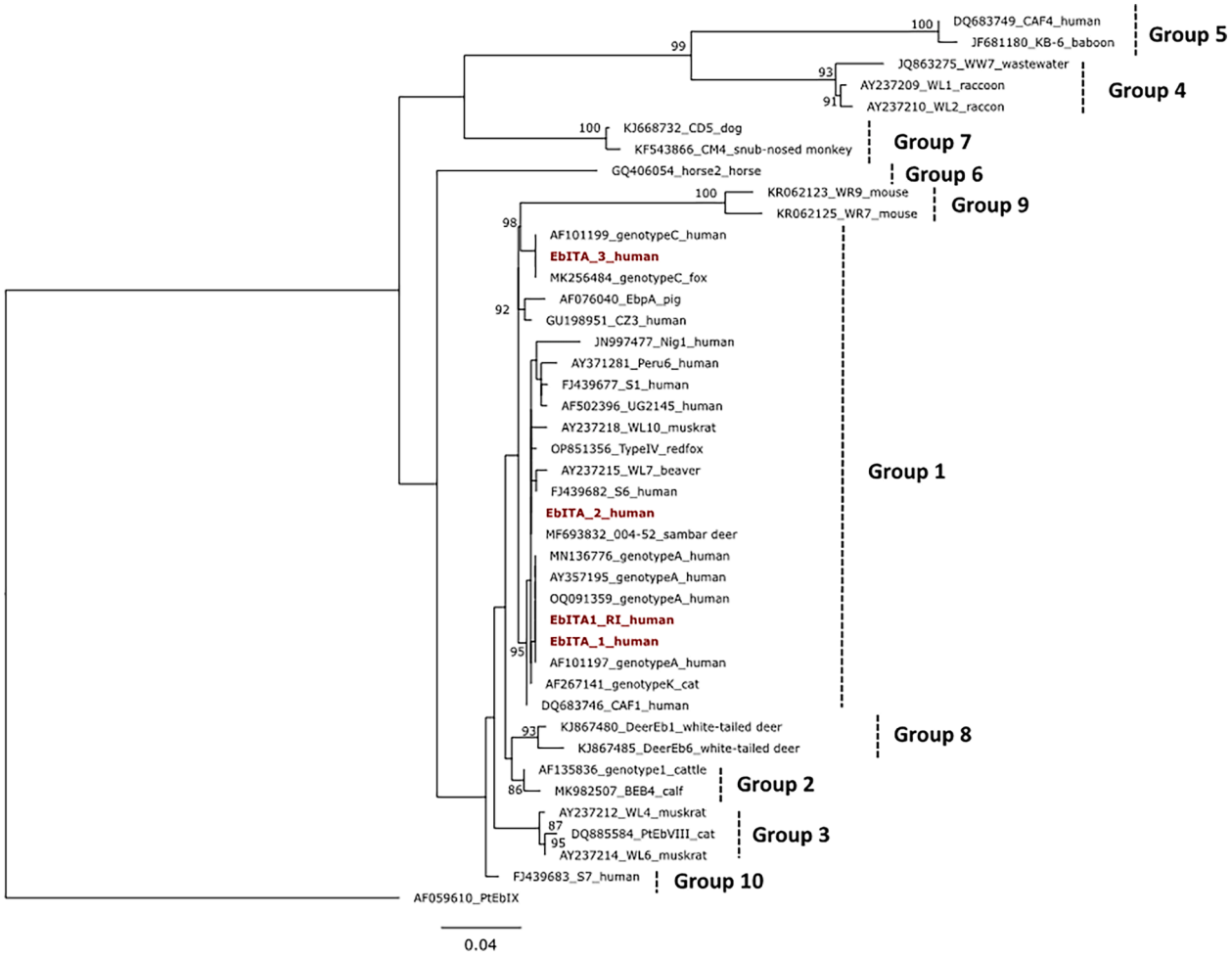

Fig. 1. *E. bieneusi* ITS rRNA phylogenetic tree inferred by the Maximum Likelihood method (ML). Sequences generated in the present study are represented in dark red and bold. Genetic distances were computed using the HKY+ G4 substitution model. Numbers on the tree nodes indicate bootstrap values >85 %. Accession numbers of sequences retrieved from GenBank are indicated together with their genotype name, host and clustered in groups.

- isolate EbITA_3 was identified as genotype C (syn. genotype TypeII), showing 100 % of identity with sequence AF101199 (100 % query cover).

Phylogenetic analysis (Fig. 1) confirmed genotype identities retrieved from Genbank, clustering all the Italian isolates in the phylogenetic Group 1, which includes both human and animal hosts. Sequences obtained were deposited in GenBank under the accession numbers OR676939-OR676942.

4. Discussion

Microsporidia have been detected in a broad range of geographical locations, spanning over 92 nations, with prevalence values associated with sanitation facilities, drinkable water, animal exposure, and diagnostic techniques; among principal species that have been identified as infecting humans, *E. bieneusi* has been the most frequently reported in clinical cases (Ruan et al., 2021). The available evidence in literature indicates that *E. bieneusi* has a significant genetic diversity across several host species and different ecological environments (Li et al., 2019a,b). Human infections are mostly caused by the phylogenetic Group 1 genotypes (such as D, EbpC, Type IV, A, and Peru8) and Group 2, with few occurrences of infections caused by genotypes belonging to the other groups. Group 1 and Group 2 are likely to be the root cause of most of the zoonotic or cross-species infections (Li et al., 2019a). Many of the Groups 3–11 genotypes have a limited range of hosts and pose a small or unknown threat to public health (Li and Xiao, 2020).

Noteworthy, a major challenge in *E. bieneusi* identification is related to the controversial nomenclature concerning the different *E. bieneusi* genotypes, with the nomenclature differing among various authors. At the same time, the phylogenetic macro-groups identified could be consolidated, also through the use of different markers in addition to ITS. Considering the matter, it is important to contemplate that the genetic identity or relatedness observed in the approximately 243-bp ITS region may not provide a comprehensive representation of the broader genetic traits present within the genome of *E. bieneusi*, which spans approximately 6 megabases (Li and Xiao, 2020).

This complex scenario poses significant difficulties in determining infections origins and assessing a proper public health significance to these microsporidia. Moreover, there is a lack of commercially available treatment options that have shown a significant efficacy in managing *E. bieneusi* infections (Zhou et al., 2023). Therefore, preventing the spreading between susceptible hosts could represent a critical aspect to reduce the risk of infection.

Because of the exceedingly small size of the spore (about 1 μm) and the interference from food particles, bacteria, fungus, and other microsporidian species, specific identification of *E. bieneusi* by traditional staining techniques is a challenge (Li and Xiao, 2020). Fortunately, microsporidia diagnostics has improved over the last decade, and several in-house PCR techniques have been extensively documented, showcasing their versatility and adaptability. Additionally, many different commercially manufactured PCR kits have been developed and made available to researchers and clinicians. Some of these also have the capability to detect various other pathogens that affect the gastrointestinal tract (Moniot et al., 2022). Novodiag® Stool Parasite, a cutting-edge diagnostic tool designed to detect the presence of nucleic acid markers associated with the identification of prevalent protozoan, helminths, and microsporidia parasites in faecal samples, offers a reliable and efficient means of identifying these common pathogens.

As for Italy, up to now, only few studies have been conducted regarding *E. bieneusi* in humans, all of them related to HIV patients and by microscopy. The first case report dates to 1995, by Caramello et al., followed by four studies between surveys, comparative diagnostics, retrospective studies and followed up treatments' controls, of which the most recent was published in the year 2000, by Maggi et al. (for details see Table 1a in Supplementary files). Due to this, we can report the first information about *E. bieneusi* genotypes from humans in Italy.

The three different genotypes here identified, respectively A, C and K, belongs all to the phylogenetic Group 1, a complex and broad phylogenetic group, which includes more than 300 genotypes isolated from almost every species of the currently known hosts for *E. bieneusi*, demonstrating the potential for a zoonotic or inter-species transmission. However, some genotypes belonging to Group 1 exhibit a notable degree of host specificity. For instance, genotypes B and C have been observed exclusively in human infections across various geographic regions (Li and Xiao, 2020). Similarly, genotype EbpB has been found solely in pig infections (Li and Xiao, 2020). Therefore, due to the lack of patients' background information, in the present study resulted challenging to assume the isolates transmission routes and/or sources of infection origin. An interesting food for thought is given by the recurrent positivity for the patient infected with genotype A. Having no access to individuals' personal information or a direct contact with the patient himself makes difficult to clearly understand the reasons for this prolonged positivity. Hardly attributable to a re-infection, since the two isolates obtained were identical each other, it can be more easily linked to drug resistance or to an unfitting therapy.

In conclusion, this investigation contributes novel information regarding the occurrence and molecular diversity of *E. bieneusi* in symptomatic individuals from Italy. The utilization of manufactured PCR kits methodologies facilitates a swift identification of this microsporidia and serves as a reliable diagnostic alternative to conventional techniques like microscopy. To mitigate the transmission of *E. bieneusi,* it is imperative to implement preventive measures within the framework of One Health. These measures aim to minimize the proximity between infected and susceptible hosts, as well as the potential contamination of food and water sources. Further investigations from a One Health perspective will be needed worldwide on human populations, animal populations, and environmental sources to better understand *E. bieneusi* zoonotic potential and public health implications.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data statment

The data that support the findings of this study are available in the public database GenBank [https://www.ncbi.nlm.nih.gov/nucleotide/].

CRediT authorship contribution statement

Isabel Guadano-Procesi: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Federica Berrilli:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Data curation. **David Di Cave:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have shared my data in the manuscript and in the additional file.

Acknowledgment

We would like to thank Dr. David Carmena, Dr. Alejandro Dashti and Dr. Ana Figueiredo for their generosity and expertise, which strongly improved this study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2024.107136.

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