Laboratory culture and life cycle of Thelazia callipaeda in intermediate and definitive hosts

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Abstract

Background: Human thelaziasis caused by *Thelazia callipaeda* (Spirurida, Thelaziidae) is being increasingly reported worldwide. Notably, it shows an epidemic trend in Guizhou Province, Southwest China. Whether *Phortica okadai* distributed in Southwest China can act as a vector of *T. callipaeda* and human-derived *T. callipaeda* animal infections have not been widely reported, there is also limited literature on animal models with *T. callipaeda* infection.

Methods: *T. callipaeda* was isolated from cases of human infection and identified by morphological and molecular analyses. *P. okadai* was cultured in a laboratory and experimentally infected with first-stage larvae (L1) collected from adult worms. Dead *P. okadai* were subjected to PCR assay and dissected every two days to detect *T. callipaeda*. Live flies were then used to infect a rabbit using a special device. The infection procedures were performed once a day (20 min) for two weeks. The infected rabbit was examined every two days for worms in the eyes.

Results: The nematode causing parasitic infection in the patient's eyes was identified as *T. callipaeda*. L1 collected from adult *T. callipaeda* successfully parasitized *P. okadai* captured in Zunyi, a city in Southwest China, and developed into L3. Experimentally infected *P. okadai*, as a vector of *T. callipaeda*, could then be used to transmit L3 to rabbits. A rabbit was successfully infected with *T. callipaeda* using *P. okadai* as the intermediate host.

Conclusions: The present study demonstrates a human-derived *T. callipaeda* infection in rabbits, through *P. okadai*, under laboratory conditions for the first time. These results indicate that there is a transmission cycle of *T. callipaeda* and suggest a likely epidemic risk due to an increasing trend of this zoonotic nematode in southwest China.

Background

*Thelazia callipaeda* (Spirurida: Thelaziidae) is a vector-borne zoonotic nematode of public health concern because it can infect a wide range of host species, including domestic and wild carnivores, lagomorphs, and humans [1]. *T. callipaeda* has long been referred to as “oriental eyeworm” due to its occurrence in humans and dogs from the Russian Federation and the Far East (i.e., Indonesia, Thailand, China, Korea, Myanmar, India, and Japan) [2]. Adult worms live in the conjunctiva, under the lids, and on the nictitating membrane. After mating, female eyeworms produce first-stage (L1) larvae, which are released in lachrymal secretions. L1 larvae are ingested by secretophagous flies, undergo two molts to the third-stage (L3) larvae in the vector testes, and then migrate to the vector proboscis. Infectious L3 larvae are transmitted to a new host during the fly's next feeding on lachrymal secretions. The development of L3 larvae into adult worms takes place in approximately 35 d [3]. Both larvae and adults of *T. callipaeda* are involved in the pathogenesis of ocular thelaziasis. Its clinical manifestations may include blepharospasm, lacrimation, congestion, discharge, epiphora, conjunctivitis, corneal edema, keratitis, and corneal ulceration [4]. A fly species that feeds on fruits, vegetables, and lachrymal secretions, *Phortica*
**Materials And Methods**

**T. callipaeda collection and identification**

A 67-year-old man from a rural area (BiJie) in Guizhou province (26 57’ 27" N, 107 21’ 38" E) southwest of China visited his doctor at the Hospital of Zunyi Medical University in September 2021 with a history of foreign body sensation, conjunctival hyperemia, and increased secretions from the eyes. Several nematode specimens were collected by an ophthalmologist using intraocular forceps, under anesthesia.
Morphological examination was performed directly under a microscope (Olympus BX43, Japan) and then, the molecular identification was performed. Briefly, genomic DNA was extracted from whole worms using a genomic DNA kit (M5 Hiper Universal DNA Mini Kit, MF033-plus, Zomanbio, China). A partial cox1 sequence of *T. callipaeda* (accession number: AB538283), approximately 200 bp in size, was amplified using a conserved primer (F: 5'AGATGGCGTTTCCTCGTCT 3'; R: 5' GCAAGAACCAATACCCACAG 3'). Amplicons were amplified using a PCR expansion kit (2xTaq Plus PCR MasterMix, TIANGEN Biotech Co, Germany), and sequenced using an ABI3730XL DNA Analyzer (Applied Biosystems, USA). Genomic DNA (1 μl) was added to the PCR reaction mix (24 μl) containing 9.5 μl nuclease-free water, 1 μl of each primer and 12.5 μl Premix (2x). The PCR reaction system used the following cycling protocol: 94 ℃ for 3 min, followed by 32 cycles of 94 ℃ for 30 s; 55 ℃ for 30 s; 72 ℃ for 60 s; followed by 72 ℃ for 5 min, and storage at 4 ℃. The obtained sequence was subjected to genetic analyses using available sequences of related nematodes from GenBank [16–18].

**P. okadai** laboratory culture

*P. okadai* captured in Zunyi, a city in southwest of China, has been identified by Huang et al in 2017 [19], and has typical morphological features, such as 3 dark bands on the legs, a white ring around the eyes and a "mountain" shaped black horizontal band on the dorsal side of the 3rd–5th abdominal segments (Fig. 2) [20]. *P. okadai* was captured and then cultured in the laboratory of Zunyi Medical University within a well-sealed cage (22 ´ 22 ´ 27 cm) at 28 ± 2 ℃, 75% ± 10% humidity, and 12/12 light/dark cycle. Fruit (pear) was fermented for three days and water was changed daily.

**P. okadai** infection procedure design and examination

First-stage larvae were squeezed out of the mature female worms and placed on a slide with a drop of saline solution and observed under the light microscope (OLYMPUS DP260, Japan).

After collection, L1 were transferred to a concave slide with a few drops of water and three-day-old, fermented pear juice (water-pear juice 1:1). Mature *P. okadai* (n=100, F:M=1:1), with food and water restricted 4 h prior, were used for experimental infection, and then the slide with L1 in the above medium was placed in well-sealed cages (22 ´ 22´ 27 cm) at 28 ± 2 ℃, 75% ± 10% humidity, and 12/12 light/dark cycle. After 20 min, 1 ml of the medium was added to attract *P. okadai* to the feed. This process lasted for two hours, after which the flies were fed normally (Additional File 1: Video S1).

Otranto et al. reported a procedure in which live and dead *P. okadai* were randomly collected every two days, examined by dissection, and subjected to molecular analysis with cox10 [21], as described above, until a positive infection was detected (Fig. 3). Flies were dissected by stretching the proboscis of *P. okadai* to detect infective L3 followed by progressively dissecting the head, thorax, and abdomen to detect the presence/absence of other developmental stages of eyeworms.

**Rabbit infection design procedures and confirmation**
Approximately 4 h before experimentally infecting the female rabbit (6 weeks, 2.6 kg), the infected *P. okadai* were starved and dehydrated. To simulate the natural environment in which the infective third-stage (L3) larvae rests on the eyes of humans or other animals through *P. okadai*, the infected stage larvae crawled out of the *P. okadai* mouthparts and parasitized the conjunctival sac of the host in the laboratory environment. We designed a device with a cage (22´ 22´ 27 cm) on the left and a fixed the rabbit’s body on the right (24´ 18´ 18 cm) (Fig. 4).

A small amount of fermented juice was added to the rabbit’s eyes and surroundings to lure the *P. okadai* (Additional File 1: Video S1). The infection procedures were performed once a day (20 min each time), with appropriate breaks or stops in the middle, depending on the rabbit’s response. The infection process lasted for two weeks, and the rabbit was observed daily for *T. callipaeda* worms. During this period, dead fruit flies were collected and dissected every two days and checked.

**Results**

*T. callipaeda* morphological identification

Eleven worms were collected from the patient’s eyes (n = 11, F:M = 7:4) (Fig. 5a, Additional file 1: Video S1). The cuticle of their body wall showed a transparent spiral with a visible internal digestive tract under a light microscope (Fig. 5b). The anterior end of the adult *T. callipaeda* has a polygonal oral sac with an elongated digestive tract and serrated cuticular striations. Coiled larvae in the twin-tube uterus were visible in the lower part of the head (Fig. 5c). The tail of the female was straight and that of the male was ventrally curved with several pairs of papillae in front of the anus (Fig. 5d). The worms were identified as *T. callipaeda* based on the key morphological features described by Rolbiecki [22, 23]. Four male and seven female *T. callipaeda* specimens were collected.

Molecular analysis

We amplified the *cox1* gene of the worm and it was identified by 2% agarose gel electrophoresis, which resulted in the appearance of a target DNA band of approximately 200 bp in length of the expected consistent size (Fig. 6). The worm was also identified as *T. callipaeda*.

Sequencing results showed that the characteristic *cox1* gene was approximately 199 bp. Alignments were compared with the *cox1* sequence available for Caenorhabditis elegans (accession no. AY171197). Because the gene sequence was only 199 bp, it was not possible to determine the specific type of the *T. callipaeda*, but by analyzing the data (Additional file 2: Table S1), it could be inferred that the genotypes might belong to h3, h7, h15, h16, h18, h19, h20, and h21 (only h7 in Korea, others in China). This *cox1* gene was comparable with European *T. callipaeda* haplotypes (h1 AM042549) at 97.4% and with Japanese *T. callipaeda* homology (h9-h12) at 97.4–99.4%.

**Morphological characteristics of L1**
Due to artificial extrusion, numerous newborn larvae and larvae curled inside the capsule were visible under the microscope (Fig. 7a). The L1 larvae had a blunt and rounded head, a slender and pointed tail, with a visible mouth capsule, and a complete digestive tract; the annulus had not yet started to form, with a size of approximately (100–120) ×5 µm (Fig. 7b). The larvae were wrapped in a curly shape in the oocyst, and the follicle size was approximately 40–50 µm (Fig. 7c; Additional file 1: Video S1); however, at this point, the larvae developed slower than the larvae follicle in a semi-ruptured state (Fig. 7d) and were smaller in size.

**Larval development in** *P. okadai*

The period in which individuals of *P. okadai* resulted positive for PCR and dissection is shown in Fig. 3. On the 4th day following infection, the PCR results were positive, demonstrating the successful infection of *P. okadai* by *T. callipaeda* L1 larvae. One larva was micro dissected from a female *P. okadai* on the 18th day, with an elongated, transparent body, approximately 1294 µm × 30 µm in size, a serrated fold slightly under the head end and rounded (Fig. 3, Fig. 8a; Additional file 1: Video S1), blunt tail end with short copulatory spines faintly visible under high-power microscopy (Fig. 8b). Ten (17.9%, n = 10, F:M = 8:2) out of 56 *P. okadai* dissected were found to be infected with *T. callipaeda* L3, from the 18th to the 30th day (Additional file 2: Table S2).

**Rabbit infection is achievable**

On the 12th day after the start of the rabbit infections, a *T. callipaeda* was found in the right eye of the rabbit (Fig. 9; Additional file 1: Video S1). *T. callipaeda* was transparent, mostly hidden under the third eyelid, and swam freely in the conjunctival fornix. 25 days later, it reached adult size, and as it grew, there was no obvious inflammation in the rabbit’s eye.

**Discussion**

To the best of our knowledge, it is impossible to mimic parasite biology in a human host in vitro or to obtain all the life stages, especially of reproductive worms. Therefore, it is necessary to establish a suitable animal model for studying *T. callipaeda*. This study established a rabbit model for *T. callipaeda* infection using L1 isolates from a human patient. This effort makes a significant contribution to the development of an animal model for *T. callipaeda* infection.

We successfully established the life cycle of *T. callipaeda* by using a rabbit and *P. okadai*. *T. callipaeda* parasites were not only identified by morphology but also by DNA sequence analysis of the *cox1* gene. The molecular analysis results revealed 97.4% homology with *T. callipaeda* haplotypes (h1) found in Europe [24] and 100% homology with the worms in China and Korea [25]. *T. callipaeda* can be cross-infected among animals with the vector *P. okadai* [26, 27]. Despite being a laboratory trial infection, this study is the first to report *T. callipaeda* from human parasites infecting animals, contrary to earlier findings.
One of the major issues concerning thelaziosis associated with *T. callipaeda* is the identification of the intermediate host, which includes not only the insect species that act as a vector but also the period of larval development in the flies. The data from experimental infestation, shown in (Figs. 3 and 8), showed *T. callipaeda* using PCR on the 4th day of *P. okadai* infection and by dissecting the flies on the 18th day. These results are consistent with those of Otranto et al. [21]. The infection rate of the male *P. okadai* was greater than that of females, which is consistent with the results of Wang et al. [28]. Infected *P. okadai* mostly contained one larva, but some were also found to contain five larvae.

Temperature affects the rate of *P. okadai* infection and the development of L1 to L3. Wang et al. [28] has shown that, under the condition of 23.4–29.7 °C, the infection rate of Drosophila was the highest (53.49%), and the developmental cycle of L1-L3 was the shortest (14 days). Ten (17.9%, n = 10, F:M = 8:2) out of 56 *P. okadai* dissected were found to be infected with *T. callipaeda* L3, from the 18th to the 30th day of our study. Different stages of larval developmental were found. This discrepancy could be explained by the fact that in Wang’s research, flies were dissected every day to retrieve all larval stages, whereas in our study flies were dissected only on specific dates to retrieve infective L3.

It is worth noting that the number of *P. okadai* that died after being infected for 20 days gradually increased. Almost no L3 larvae were found in dead *P. okadai* after 25 days, and dissected flies revealed that the L3 larvae were so large that it was difficult for them to burst out from the proboscis of *P. okadai*. Therefore, we estimated that the large body size of L3 larvae interfered with feeding, resulting in the death of *P. okadai*. This may also explain why only one *T. callipaeda* was found in the natural environment simulated in this experiment. Previous research has shown that parasites are difficult to spot when they are in the larval stages or when there are few of them [2]. Accordingly, diagnosis was achieved by direct visualization of the worms in both eyes of the infected rabbit (Additional file 1: Video S1). This work provides evidence that *P. okadai*, collected in Guizhou Province and experimentally infected with L1 of *T. callipaeda*, may act as a vector for this nematode in Southwest China. Meanwhile, the current protocol for rearing *P. okadai* under laboratory conditions in the present study represents a useful tool for morphological and behavioral investigations such as “lachryphagy.”

Over the past three years, the COVID-19 pandemic has taught the relevance of integrative medicine (also known as One Health) to scientists, politicians, and lay men, increasing their awareness of the connection between medical and veterinary sciences [29]. Thelaziasis caused by *T. callipaeda*, as a vector borne zoonotic parasitic disease, has also received a growing interest. *T. callipaeda* shares the same environment and lives with its hosts [30]. However, with urbanization, invasion of wildlife into urban areas, animal movements between regions, countries, and continents, as well as human leisure activities, diverse wildlife-domestic animal human interfaces have been created, and the prevention and control of *T. callipaeda* are proving to be challenging [31].

China covers almost all ecosystems in the world, and many native or non-native species have suitable habitats [32]. In addition to rapid economic development, implementation of the "Silk Road Economic Belt" strategy has also greatly increased the risk of *T. callipaeda* transmission. Until now, except for
milbemycin oxime, there have been no other effective drugs to prevent the infection of *T. callipaeda* in endemic areas [33–35]. Therefore, the development of novel drugs and treatment strategies is necessary. This rabbit model provides an important platform for anti-*T. callipaeda* drug research in a preclinical setting and provides tools for future studies of immune evasion mechanisms of *T. callipaeda*.

Collectively, our findings suggest that *T. callipaeda* can be transmitted from humans to animals as well as that the L1 collected from female *T. callipaeda* is also infectious to *P. okadai* captured in Southwest China. The methods used provides extensive contributions to establishing a suitable animal model for *T. callipaeda* infection.

**Conclusions**

In this study, we successfully established a rabbit model of *T. callipaeda* using the vector *P. okadai*. To the best of our knowledge, the present study is the first to demonstrate human-derived *T. callipaeda* rabbit infection through *P. okadai* under laboratory conditions. These results indicate that there is a transmission cycle of *T. callipaeda* and suggest a likely epidemic risk and an ongoing expanding trend of this zoonotic nematode in southwest China. The methods used in this study provide a reference for establishing other animal models of *T. callipaeda*. The establishment of a rabbit model also provides an important platform for exploring the immune escape mechanism of *T. callipaeda* and a tool for developing novel drugs and treatment strategies for thelaziasis.

**Abbreviations**

PCR: polymerase chain reaction; L1: first stage; L3: third stage; *cox1*: mitochondrial cytochrome c oxidase subunit 1 gene; F:M= female: male.

**Declarations**

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**Availability of data and materials**
The data supporting the conclusions of this study are included in the article. The raw datasets used and/or analyzed in the present study are available upon request. Datasets supporting the conclusions of this study are included in this article. The sequences obtained during the current study are available in the GenBank database.

Authors’ contributions

LH, WLJ, THR, and LD devised the study. LH, WLJ, THR, and LD were responsible for the collection of the wildlife thelaziasis cases used in this study. LD and CZY obtained the sequence of *T. callipaeda* and analyzed the results. All members participated in *P. okadai* capture. WLJ and LD drafted the manuscript and all authors critically contributed to the final version. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal study and the use of samples from patients were approved by the ethical commission of Zunyi Medical University, China, and written informed consent was obtained from the patient at the time of the samples collection.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References


Figures

Fig. 1

Figure 1

Map of human thelaziasis and P. okadai distribution in China. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city, or area or of its
authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors

**Figure 2**

The typical morphological features of *P. okadai*. (a) Abdomen of *P. okadai* showing dark bands in characteristic configuration. (b) Female *P. okadai* in lateral view. A pale ring (arrow) is seen around the red eye. (c) 3 dark bands on the legs

**Fig. 3**

- **P. okadai infection**
- **Rabbit infection**
- **Infection experiment ended**
- **PCR positive**
- **Positive for L3**
- **Rabbit infected successfully**
Figure 3

The infection and examination procedures (+: positive, -: negative, M: male, F: female)

Figure 4

Device used during rabbit infection. A cage on the left and a fixed rabbit body on the right
Figure 5

*T. callipaedia* morphological identification. (a) The left eye of the patient with *T. callipaedia* adult worms (n=11, F:M=7:4). (b) Adult male *T. callipaedia* show a transparent spiral with a clearly visible internal digestive tract. (c) Anterior end of adult female *T. callipaedia* showing oral sac, digestive tract and coiled larvae in the uterus. (d) Posterior end of a male *T. callipaedia* with non-protruding anal opening, post-anal papilla and short copulatory spines.
Figure 6

PCR results of \textit{T. callipaeda} \textit{cox1} (M: 1,000 bp Marker, 1 and 2: infection of \textit{P. okadai} DNA extract)
Figure 7

Morphological characteristics of L1. (a) Mid-section of adult female *T. callipaeda* showing live larvae in uterus and large numbers of newborn larvae. (b) *T. callipaeda* newborn larvae showing rounded head, a slender and pointed tail and a complete digestive tract. (c) *T. callipaeda* L1 larvae coiled within intact follicular sacs. (d) *T. callipaeda* L1 larvae breaking out of the follicular sacs
Figure 8

_P. okadai_ dissection and _T. callipaedia_ observations. (a) L3 larvae dissected from the mouthparts of _P. okadai_. (b) Anterior end of L3 larvae _T. callipaedia_ showing oral sac, digestive tract, and serrated folded body surface. (c) Posterior end of L3 larvae _T. callipaedia_ with a dorsal papilla and two lateral papillae.
Fig. 9

An adult *T. callipaeda* (arrow) in the rabbit’s eye

**Supplementary Files**

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