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# Molecular characterization of *Thelazia lacrymalis* (Nematoda, Spirurida) affecting equids: a tool for vector identification

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**Abstract**

Equine thelaziosis caused by the eyeworm *Thelazia lacrymalis* is a parasitic disease transmitted by muscid flies. Although equine thelaziosis is known to have worldwide distribution, information on the epidemiology and presence of the intermediate hosts of *T. lacrymalis* is lacking. In the present work, a PCR-RFLP based assay on the first and/or second internal transcribed spacer (ITS1 and ITS2) of ribosomal DNA was developed for the detection of *T. lacrymalis* DNA in its putative vector(s). The sensitivity of the technique was also assessed. The restriction patterns obtained readily differentiated *T. lacrymalis* from four species of *Musca* (Diptera, Muscidae) (i.e. *Musca autumnalis*, *Musca domestica*, *Musca larvipara* and *Musca osiris*), which are potential vectors of equine eyeworms. The molecular assay presented herein is a useful tool to identify the intermediate host(s) of *T. lacrymalis* in natural conditions and to study its/their ecology and epidemiology.


*Keywords:* *Thelazia lacrymalis*; Horse; *Musca*; Intermediate host; ITS1; ITS2; PCR-RFLP

## 1. Introduction

*Thelazia* (Spirurida, Thelaziidae) eyeworms are parasitic nematodes responsible for ocular infestations in several animals (e.g. canids, felids, ruminants, equids) as well as in humans [1–3]. Horse infection by *Thelazia lacrymalis* has been reported in several Asian, American and European countries (see for details [3–7]). This nematode localizes on the surface of the eye, under the eyelids and the conjunctiva, on the lachrymal gland and in its excretory ducts causing subclinical to clinical disease, with symptoms ranging from conjunctivitis, epiphora and photophobia to keratitis and ulcers; sometimes endophtalmitis and blindness may occur [2–4,8,9].

The biological cycle of *Thelazia* requires non-biting flies to act as intermediate hosts which transmit the third infective stage larvae (L3s) feeding on ocular secretions, tears and conjunctiva of animals [3,10,11].

Identification of the species of insects that may act as biological vectors of eyeworms is crucial to study the epidemiology of thelaziosis and plan prophylactic programs based on the control of specific vectors. Most investigations on eyeworm vectors in field and laboratory conditions have been carried out by dissecting muscids that act as intermediate hosts of bovine thelaziosis, i.e. *Thelazia gulosa*, *Thelazia rhodesi* and *Thelazia skrjabini* (see [12] for details). Information on the intermediate hosts of equine *T. lacrymalis* is limited and regards the finding of immature *Thelazia* spp. stages in *Musca osiris* collected around the eyes of a horse in Russia [2] and the development to infective L3s in experimentally infected *Musca autumnalis* [4].

Investigations on the fly species involved in the transmission of *Thelazia* eyeworms are often thwarted by the many constraints linked to arthropod dissection (see Section 4) which make it very difficult to identify the species acting as vectors of *Thelazia* spp.

Polymerase Chain Reaction (PCR)-based approaches that use appropriate genetic markers have recently opened

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new perspectives and provided useful tools to overcome limitations in the epidemiological study of the insect vectors through the detection of the DNA of nematodes of human and veterinary concern, as for *Onchocerca volvulus* in blackflies [13] and *Dirofilaria immitis* and *Brugia malayi* in mosquitoes [14,15].

The Internal Transcribed Spacer 1 (ITS1) of the ribosomal DNA (rDNA) of *Thelazia* spp. have proved to be particularly suitable for the detection of eyeworm DNA in their vector flies, as has recently been demonstrated for bovine eyeworms [12].

The aim of the present study was to establish the level of sequence variation in the ITS1 and in the Internal Transcribed Spacer 2 (ITS2) of *T. lacrymalis* and to evaluate the usefulness of a molecular assay for the detection and identification of *T. lacrymalis* in its putative vector(s).

## 2. Materials and methods

### 2.1. Parasite collection and DNA extraction

Eyeworms were collected from the eyes and associated tissues of native horses slaughtered at the abattoir of the municipality of Noicattaro (Apulia region, southern Italy) as previously described [7]. After washing in physiological saline (pH 7.3) the anterior and posterior ends of all nematodes were cut off and cleared in lactophenol to be identified following morphological keys [2]. The remainder of each worm was labelled with anamnestic data and frozen at −80 °C before performing molecular analyses.

Genomic DNA was extracted from fifteen individual adults of *T. lacrymalis* by using the QIAamp Tissue Kit (Qiagen GmbH, Germany) after disruption with liquid nitrogen.

Secretophagous flies were netted in farms located in a *Thelazia*-free area and identified to species [16] as follows: *M. autumnalis*, *Musca domestica*, *Musca larvipara* and *M. osiris*. Genomic DNA was extracted by using Tris–HCl/Proteinase K (20 mg/kg) treatment from the legs of 10 single specimens of each species of *Musca* collected.

### 2.2. PCR, sequencing and ITSs analysis

All the samples of DNA extracted both from the nematodes and flies were subjected to PCR protocols using four conserved primers (A, $B_1$, C, D), the rationale being to amplify the ITS1 (primer set A–C) of the nematodes and ITS2 (primer set D–$B_1$) of the nematodes and the flies [17–19]. All PCR products were subjected to electrophoresis in 2% TBE agarose gels (65 mM Tris–HCl, 27 mM boric acid, 1 mM EDTA, pH 9; Bio-Rad, Richmond, CA, USA), stained with ethidium bromide and detected using UV transillumination. Amplicons were purified over mini-columns (Ultrafree-DA, Millipore, Bedford, MA, USA) and subjected to automated sequencing (version 2; Applied Biosystems in a ABI-PRISM 377). Sequences were determined in both orientations and electropherograms verified by eye. Sequences were aligned using the program Clustal X [20] with default settings and penalties, and compared with one another and with those available in the GenBank™ database. Pairwise comparisons were made of the level of sequence differences ($D$) among all ITSs by using the formula ($D = 1 - M/L$) and criteria previously described [21,22]. In particular, $M$ is the number of alignment positions where the two taxa have a base in common and $L$ is the total number of alignment positions over which the two species are compared. Polymorphic positions in the sequence of one species were not considered to be different from those of another species if the latter contained one of the polymorphic nucleotides in its sequence, while alignment gaps were treated as equivalent to substitutions [21,23].

### 2.3. Restriction analysis

Restriction maps of the ITSs were determined using the software Webcutter 2.0. *Sca*I and *Hpa*II (Fermentas, Hanover, MD, USA) endonucleases were chosen for the diagnostic digestion of the ITS1 and ITS2 amplicons, respectively, as the former cut the *T. lacrymalis* A–C amplicons (ITS1), and the latter cut the *T. lacrymalis* D–$B_1$ amplicons (ITS2) but not the *Musca* spp. amplicons. Both endonucleases cleaved at sites where no intraspecific polymorphisms occur. The PCR-RFLP assay was performed—with slight modifications to the manufacturer's recommendations—in 20 μl total volume reaction-mix, containing 15 μl of amplicon, 10 U enzyme and the standard buffer provided by the manufacturer. Samples were digested overnight at 37 °C and, after EtBr staining, restriction patterns were visualized on a 2.8% agarose-TAE gel and compared against a 100 bp DNA ladder (New England Biolabs, Beverly, MA, USA). Where restriction fragments were produced, RFLP assays were validated at least three times.

### 2.4. Sensitivity of the assay

To assess the sensitivity of the PCR-RFLP, DNA extracted from one specimen of *T. lacrymalis* was spectrophotometrically quantified and spiked with DNA extracted from a single specimen of each of the four *Musca* species. Dilutions were made by serially titrating eyeworm DNA into fly DNA and were subjected to the PCR reactions as described above by using the A–C and D–$B_1$ primers.

## 3. Results

The primer pair A–C yielded amplicons of the expected size (∼450 bp) for the DNA of *T. lacrymalis* and, as