Veterinary Parasitology 246 (2017) 11–18

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Original research paper

First record of *Gongylonema nepalensis* in domestic and wild ruminants in Europe

A. Varcasia[a,*], A. Scala[a], A. Zidda[a], P.A. Cabras[b], G. Gaglio[c], C. Tamponi[a], A.P. Pipia[a], A. Setsuda[d], H. Sato[d,*]

[a] Laboratory of Parasitology, Veterinary Teaching Hospital, Department of Veterinary Medicine, University of Sassari, Via Vienna 2, 07100 Sassari, Sardinia, Italy
[b] Istituto Zooprofilattico Sperimentale Della Sardegna, Tortolì, Via Aresu 2, 08048 Tortolì, Ogliastra, Sardinia, Italy
[c] Parasitology Unit, Faculty of Veterinary Medicine, University of Messina, Polo Universitario Annunziata, Messina, Italy
[d] Laboratory of Parasitology, United Graduate School of Veterinary Science, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan

ARTICLE INFO

Keywords:
Gongylonema nepalensis
Ruminant
Sardinia island
Scanning electron microscopy
rDNA
cox-1
New geographical record
New host record

ABSTRACT

The gullet worm, *Gongylonema pulchrum*, is cosmopolitan in distribution, infecting a variety of mammals including domestic and wild ruminants. *Gongylonema nepalensis* recently collected from the esophageal epithelium of water buffaloes in Nepal was separated from *G. pulchrum* based on its distinctly shorter left spicule relative to body length and unique nucleotide sequences of the ribosomal RNA gene (rDNA) and the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox-1*). During meat inspections at four abattoirs on Sardinia Island, Italy, 25 *Gongylonema* worms were collected from one each individual of cattle (n = 8), sheep (n = 7), goats (n = 4), and mouflon (*Ovis aries musimon*; n = 6), and characterized morphologically and genetically. Intriguingly, all of the collected worms from these ruminants were *G. nepalensis*, exhibiting comparable body lengths to *G. pulchrum* in cattle from other regions but with significantly shorter left spicules like *G. nepalensis* (less than 20.9% of the entire body length in contrast to 21.8–65.6%, the reported proportion of *G. pulchrum*). Furthermore, the rDNA nucleotide sequences of these worms from different ruminant species on Sardinia Island were almost identical to each other and to Nepalese *G. nepalensis* isolates. With the exception of one worm from a sheep (displaying a single nucleotide substitution), the 369-bp *cox-1* nucleotide sequences of all the Sardinian *G. nepalensis* isolates from the different host sources were absolutely identical, forming a clade with Nepalese *G. nepalensis* isolates and not *G. pulchrum* isolates. The present study reveals that *G. nepalensis* is not a local parasite in the Indian subcontinent (Nepal), but instead has a certain geographical distribution in Europe and takes several ruminant species as a definitive host.

1. Introduction

The genus *Gongylonema* (Nematoda: Spiruridea: Gongylonematidae) has approximately 28 nominal species in wild and domestic mammals and nine species in birds (Yamaguti, 1961; Skrjabin et al., 1967; Ashour and Lewis, 1986; Diouf et al., 1997; Sato, 2009; Kinsella et al., 2016). *Gongylonema pulchrum* (Molin, 1857), also known as the "gullet worm" because of its localization in the epithelium of the upper digestive tract, is distributed worldwide and reported from a variety of domestic and wild mammals such as cattle, sheep, goats, donkeys, cervids, equines, camels, bears, pigs, non-human primates, and humans (Alicata, 1935; Zinter and Migaki, 1970; Lichtenfels, 1971; Kirkpatrick et al., 1986; Anderson, 1992; Jelinek and Löscher, 1994; Duncan et al., 1995; Sato et al., 2005; Zhu et al., 2012; Kheirandish et al., 2013).

Transmission of the gullet worm to definitive hosts occurs through ingestion of intermediate hosts such as infected dung beetles, or drinking water contaminated with third-stage larvae (Cappucci et al., 1982; Anderson, 1992; Kudo et al., 1996). Although parasitism of this spirurid nematode is usually a latent infection, some fatal cases of gongylonemiasis have been reported in cattle and non-human primates such as Goeldi's monkeys (Cebotarev and Poliscuk, 1959; Duncan et al., 1995; Brack, 1996; Sato et al., 2005; Adkesson et al., 2007). Human gongylonemiasis has been reported from many countries, with more than 150 cases recorded worldwide since the first reported case in Italy (Xu et al., 2000; Haruki et al., 2005; Allen and Esquela-Kerscher, 2013; Pesson et al., 2013).

By the incorporation of molecular genetic analyses based on different nucleotide sequence profiles of the nuclear ribosomal RNA gene (rDNA) and mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox-1*) into the taxonomy of *Gongylonema* worms, Setsuda et al. (2016)

* Corresponding authors.
E-mail addresses: varcasia@uniss.it (A. Varcasia), sato7dp4@yamaguchi-u.ac.jp (H. Sato).

http://dx.doi.org/10.1016/j.vetpar.2017.08.022
Received 6 July 2017; Received in revised form 28 August 2017; Accepted 29 August 2017

recently separated *G. nepalensis* from water buffaloes in Nepal from *G. pulchrum*, despite the fact that these two species had almost identical morphological features apart from a distinct difference in the relative length of the left spicule to the entire body.

Following the world's first record of human gongylonemiasis by Pane (1864), several epidemiological surveys of *Gongylonema* worms have been conducted in domestic animals in Italy (Pellegrini, 1957; Latini and Asdrubali, 1964; Macchioni and Marconcini, 1965; Restani, 1965; Arru et al., 1994), revealing a high prevalence of *G. pulchrum* in sheep and cattle in the country (38–100%). In the present study, we have morphologically and genetically characterized *Gongylonema* worms collected from cattle (*Bos taurus*), sheep (*Ovis aries*), goats (*Capra hircus*), and European mouflon (*Ovis aries musimon*) on Sardinia Island, Italy, since preliminary microscopic observations of some representative worms detected rather short left spicules relative to the entire body length as in *G. nepalensis*.

2. Materials and methods

2.1. Parasite collection and morphological examination

Full-length esophagi of domestic ruminants (cattle, sheep, and goats) were intermittently collected at four abattoirs (Nulvi in Sassari Province, Lula in Nuoro Province, Baunei in Ogliastra Province, and Arborea in Oristano Province on Sardinia Island, Italy) between March 2014 and July 2016. Full-length esophagi of multiple wild European mouflon were collected from roadkill animals at the Istituto Zooprofilattico Sperimentale Della Sardegna, Tortolì in Ogliastra Province in the same island. The mucosal surface was carefully checked with the naked eye, and individual worms were removed from the esophageal epithelium using fine forceps and fixed in 70% ethanol. After microscopic observation and measurements, all the worms were stored at −20° C with the exception of three worms (two males and one female) selected for scanning electron microscopy (SEM).

For SEM, specimens were dehydrated through a graded series of ethanol from 70% to 100%, freeze-dried with liquid CO_2 according to the critical point method, and mounted onto stubs. Mounted specimens were then sputter-coated with a palladium gold layer (20 nm ± 5%) and observed with an SEM Zeiss EVO 10 MA (Carl Zeiss Microscopy GmbH, Jena, Germany).

Collected specimens, excluding portions used for SEM (three worms) or DNA extraction (11 worms), were deposited in the Parasite Collection of the Institute of Parasitology and Parasitic Disease of the University of Sassari, Italy, under specimen numbers GONSA001–GONSA025.

2.2. DNA extraction, polymerase chain reaction (PCR), and sequencing

Parasite DNA was individually extracted from 11 worms (three from cattle, five from sheep, one from a goat, and two from mouflon) using a commercial kit and following the manufacturer's instructions (PureLink® Genomic DNA Mini Kit; Invitrogen, Carlsbad, California, USA). PCR amplification of overlapping rDNA fragments was performed in a 25-μl volume using different primer combinations as previously described (Makouloutou et al., 2013a). The *cox-1* region of *G. pulchrum* mitochondrial DNA (mtDNA) was amplified by a combination of new primers, Gpul Cox1-F (5′-GTGGTTTTGGTAATTGAATGCTA-3′) and Gpul Cox1-R (5′-ATGAAAATGTGCCACTACATAATATGTATC-3′), which were designed using online software 'Primer3web ver.4.0.0′ (Untergasser et al., 2012) referring to a *cox-1* sequence of *G. pulchrum* (DDBJ/EMBL/GenBank accession no. KM264298). The PCR cycling protocol was 3 min at 94° C, followed by 40 cycles at 94 °C for 45 s, 52 °C for 1 min, and 72 °C for 1 min, then a final extension at 72 °C for 7 min.

PCR products were purified using a commercial kit, NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. KG, Düren, North Rhine-Westphalia, Germany). Following direct sequencing of PCR amplicons, sequences were assembled manually with the aid of the CLUSTAL W multiple alignment program (Thompson et al., 1994), and analyzed by using the Basic Local Alignment Search Tool (BLAST) available on the NCBI homepage (https://blast.ncbi.nlm.nih.gov/Blast.cgi.). For rDNA segments containing the internal transcribed spacer (ITS) region, the amplicon was cloned into a plasmid vector, pTA2 (Target Clone™; TOYOBO, Dojima Hama, Osaka, Japan), and transformed into *Escherichia coli* JM109 (TOYOBO) according to the manufacturer's instructions. Following propagation, the plasmid DNA was extracted using a FastGene Plasmid Mini Kit (NIPPON Genetics Co., Tokyo, Japan), and inserts from three independent clones were sequenced using universal M13 forward and reverse primers.

The nucleotide sequences reported in the present study are available from the DDBJ/EMBL/GenBank databases under the accession numbers LC278392–LC278394.

2.3. Phylogenetic analysis

For phylogenetic analysis, the newly obtained *cox-1* sequences, 369 bp in length, of *Gongylonema* worms collected in the present study and those of the same genus retrieved from the DDBJ/EMBL/GenBank databases were used. The accession numbers of the sequences analyzed in the present study are given in the figure showing the phylogenetic trees. Maximum likelihood (ML) analysis was performed with the program PhyML (Guindon and Gascuel, 2003; Dereeper et al., 2008) provided on the 'phylogeny.fr' website (http://www.phylogeny.fr/). The probability of inferred branches was assessed by the approximate likelihood ratio test (aLRT), an alternative to the non-parametric bootstrap estimation of branch support (Anisimova and Gascuel, 2006).

3. Results

3.1. Morphological examination by light microscopy

Twenty-five worms in total were collected from one steer (one male and seven female worms), one sheep (three male and four female worms), one goat (two male and two female worms), and two mouflon (three male and three female worms). Long slender worms were embedded in a zig-zag manner in the esophageal mucosa. The males were 34–46 mm in length and 0.18–0.31 mm in width, and the females were 80–120 mm in length and 0.30–0.44 mm in width. The anterior portion of male and female worms was characterized by prominent cuticular bosses with symmetrical lateral alae on both sides. The mouth had small dorsal and ventral lips, connecting to the muscular and long glandular portions of the esophagus. The posterior portion of male worms was characterized by asymmetrical caudal alae with four to six pairs of papillae, and distinctly longer left spicules and short right spicules. The caudal end of female worms was bluntly conical, and the vulva situated relatively close to the posterior end. Two uterine branches were full of a great number of oval embryonated eggs measuring 0.052–0.055 mm by 0.029–0.032 mm. The morphological features including the measurements of all collected nematodes were identical regardless of host species, resembling either *G. pulchrum* or *G. nepalensis* (Table 1). The relative length of the left spicule to the entire body length of the collected worms in the present study was 17.3–20.9%, corresponding to that of *G. nepalensis* (15.6–21.1%), distinct from that of *G. pulchrum* (21.8–65.6%), according to Setsuda et al. (2016).

3.2. Morphological examination by SEM

The anterior end of male and female worms was covered by cuticular bosses variable in shape and size, having two lateral alae with some breaks along their length (Fig. 1a, c). Anterior to each ala, a lateral cervical papilla was localized (Fig. 1a, b). The oral opening was dorsoventrally elongated and bordered by a cuticular circumoral