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Molecular identification of the rumen flukes *Paramphistomum leydeni* and *Paramphistomum cervi* in a concurrent infection of the red deer *Cervus elaphus*

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Abstract

Paramphistomosis, caused by paramphistomid flukes, is a gastrointestinal parasitic disease of domestic and wild ruminants. Originally thought to be limited to the tropics and subtropics, the disease has recently been reported in temperate regions. Here we describe the concurrent infection of a red deer doe (*Cervus elaphus*) with *Paramphistomum leydeni* and *Paramphistomum cervi*. This is the first report of *P. leydeni* in Croatia. Flukes were identified on the basis of morphological keys (tegumental papillae) and sequencing of the internal transcribed spacer region 2 in ribosomal DNA. Our results confirm that the absence of tegumental papillae allows *P. cervi* to be differentiated morphologically from other paramphistomid species in Europe based on incident light stereomicroscopy. Nevertheless the limitations of morphological identification and taxonomic issues suggest that previous findings on paramphistomid infection should be interpreted carefully. The possible worldwide distribution of these pathogens means that paramphistomosis may be more common and its economic impact greater than previously thought.

Introduction

Paramphistomosis (or paramphistomiasis) is a gastrointestinal parasitic disease caused by digenean trematodes of the Paramphistomidae family. Paramphistomid flukes have a dixenous life cycle with aquatic snails as intermediate hosts, while domestic and wild ruminants serve as definitive hosts. Adult flukes parasitize the fore stomachs, causing mild disease that occasionally manifests as rumen inflammation, irregular rumination and wasting. Much more severe symptoms are caused by juvenile flukes as they migrate through the intestines and parasitize the submucosa of the duodenum, feeding on epithelial cells. This results in fetid diarrhoea, electrolyte and protein loss, generalized oedema, anorexia and, in rare cases, anaemia (Sanabria & Romero, 2008). Paramphistomosis has been described in lowland and frequently flooded habitats, around lakes and marshlands (Sanabria & Romero, 2008). Originally the disease was thought to be limited to the tropics and subtropics (Taylor *et al.*, 2007), but recent studies have detected it in temperate regions (Nikander & Saari, 2007).

Many Paramphistomidae species were initially described based purely on morphology, primarily the morphology of the acetabulum, pharynx, terminal genitalium, tegumental papillae and internal organs (Eduardo, 1982a). However, the facts that flukes have thick, robust bodies and that most specimens from the gastrointestinal tract are sexually immature make morphological identification less reliable. As a result, some of the previously identified species are likely to be synonymous. The taxonomy of Paramphistomidae began to undergo major revision once sequencing of the internal transcribed spacer region 2 (ITS2) of ribosomal DNA came into use for species identification

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(Bazsalovicsová *et al.*, 2010; Lotfy *et al.*, 2010; Sanabria *et al.*, 2011; Ma *et al.*, 2015). For instance, whether *Paramphistomum leydeni* and *P. cervi* were one or two species remained controversial until 2015, when analysis of mitochondrial DNA ITS regions of ribosomal DNA proved them to be distinct (Ma *et al.*, 2015).

The epidemiology of *Paramphistomum* flukes in Croatia is poorly understood, since only a few reports have been published, which have described ruminal flukes in domestic and wild ruminants. In Europe, ruminal flukes infecting red deer (*Cervus elaphus*) have been studied in Slovakia, Serbia and Ireland, with different species identified in each country: *P. cervi* in Slovakia (Bazsalovicsová *et al.*, 2010), *P. microbothrium* in Serbia (Pavlović *et al.*, 2012) and *P. leydeni* in Ireland (O'Toole *et al.*, 2014). O'Toole *et al.* (2014) also identified *P. leydeni* in Irish fallow deer (*Dama dama*). Of those previous case reports from Croatia and three other studies from European countries, only Bazsalovicsová *et al.* (2010) and O'Toole *et al.* (2014) used molecular methods for species identification.

In the present study we describe concurrent infection of two species of paramphistomes in the rumen of a red deer together with the occurrence of the liver fluke (*Fascioloides magna*). This is the first molecular identification of paramphistomid species in Croatia, and the first report of *P. leydeni* in the country.

Materials and methods

A red deer doe was shot during regular game management near Lipovljani, in central Croatia. As part of a wildlife health monitoring programme, the digestive system and liver were transported to the Faculty of Veterinary Medicine at the University of Zagreb for parasitological examination. The gastrointestinal tract was opened, flushed with water and investigated for endoparasites. Contents of the stomach and intestine were mixed with water, sedimented and examined under a stereomicroscope. Faeces from the rectum were analysed using flotation in saturated $ZnSO_4$ solution (specific gravity 1.35).

Flukes found in the rumen were counted, washed with phosphate-buffered saline and stored in 96% ethanol. Species were identified using both morphological and molecular tools. Randomly chosen ruminal flukes were air-dried, examined under the stereomicroscope with incident illumination, and assigned to two groups based on the presence of tegumental papillae (Eduardo, 1982a). Flukes were cut into two parts, pressed and checked for the presence of eggs under the stereomicroscope.

Then DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA). The ITS2 sequence was amplified by polymerase chain reaction (PCR) using the primers GA1 (Anderson & Barker, 1998) and BD2 (Luton *et al.*, 1992). PCR reactions (25 µl) consisted of 5 µl of DNA, 12.5 µl of GoTaq® Hot Start Colorless Master Mix (Promega), 5.5 µl of H_2O and 0.2 mM of each primer. PCR conditions were 2 min at 95°C; 35 cycles at 94°C for 30 s, 55°C for 1 min and 72°C for 30 s; and a final elongation at 72°C for 10 min. Amplicons were purified and sequenced by Macrogen Europe (Amsterdam, The Netherlands). Sequence alignment was performed using Clustal W (Thompson *et al.*, 1994) as implemented in BioEdit software (Hall, 1999). Alignments were checked manually and compared with sequences available in GenBank, using BLAST.

The liver was examined macroscopically from the outside and then cut into slices 2 cm thick, which were flushed with water and examined for immature and mature flukes (*F. magna*), cysts and migratory channels. All parasites were collected and counted.

Results and discussion

The ruminal walls of the red deer were covered with 2719 ruminal flukes. Sixty-one parasites, chosen randomly, were examined under the stereomicroscope and separated into two groups on the basis of the absence of tegumental papillae (*P. cervi* type, 2 of 61 flukes) or their presence (other paramphistomid types, 59 of 61 flukes) (fig. 1). Eggs were found in all flukes examined. Liver examination revealed 54 specimens of liver fluke (*F. magna*). Coprological examination revealed individual lungworm larvae (Protostrongylidae), strongylid and paramphistomid eggs in the faeces.

Amplification of DNA extracted from 61 ruminal flukes led to a 320-bp product. In 59 samples, the sequence was identical to a *P. leydeni* sequence deposited in GenBank, and the corresponding fluke was identified as paramphistomid type under the microscope (table 1). The remaining two samples were identical to *P. cervi* sequences deposited in GenBank and the corresponding flukes were identified as *P. cervi* type. The *P. leydeni* and *P. cervi* sequences differed at nine polymorphic sites and were deposited in GenBank under accession numbers KX274232 and KX274233. The *P. leydeni* sequences in this study matched those isolated from goats from China (Ma *et al.*, 2015), cattle from Uruguay (unpublished), cattle from Argentina (Sanabria *et al.*, 2011) and fallow deer from Ireland (unpublished). The *P. cervi* sequences in this study matched those isolated from sheep from China (Zheng *et al.*, 2014) and red deer from Slovakia (Bazsalovicsová *et al.*, 2010).

Here we used both morphological and molecular methods to analyse red deer ruminal flukes, revealing concurrent *P. leydeni* and *P. cervi* infection in a red deer with fascioloidosis. We also confirmed that the absence of tegumental papillae can be used to differentiate *P. cervi* from other paramphistomid species in Europe, using incident light stereomicroscopy of air-dried flukes. This approach may not be appropriate in other parts of the world, where the existence of other paramphistomid flukes without tegumental papillae may result in misdiagnosis. Examples of other flukes without papillae include *Paramphistomum cephalophi*, reported so far only in the small intestine of a black-fronted duiker (*Cephalophus nigrifrons*) in Africa (Eduardo, 1982b), *Cotylophoron macrosphinctris*, reported in the rumen of African buffalo (*Bubalus* (*Syncerus*) *caffer*) (Eduardo, 1985), *Gigantocotyle gigantocotyle*, reported in the stomach of common hippopotamus (*Hippopotamus amphibius*) and *Gigantocotyle duplicitestorum*, reported in the stomach and small intestine of *H. amphibius* in Africa (Eduardo, 1984). It may also be possible to differentiate *P. cervi* from other 'non-papillar' flukes using data on the geographical distribution of flukes and hosts, as well as additional morphological

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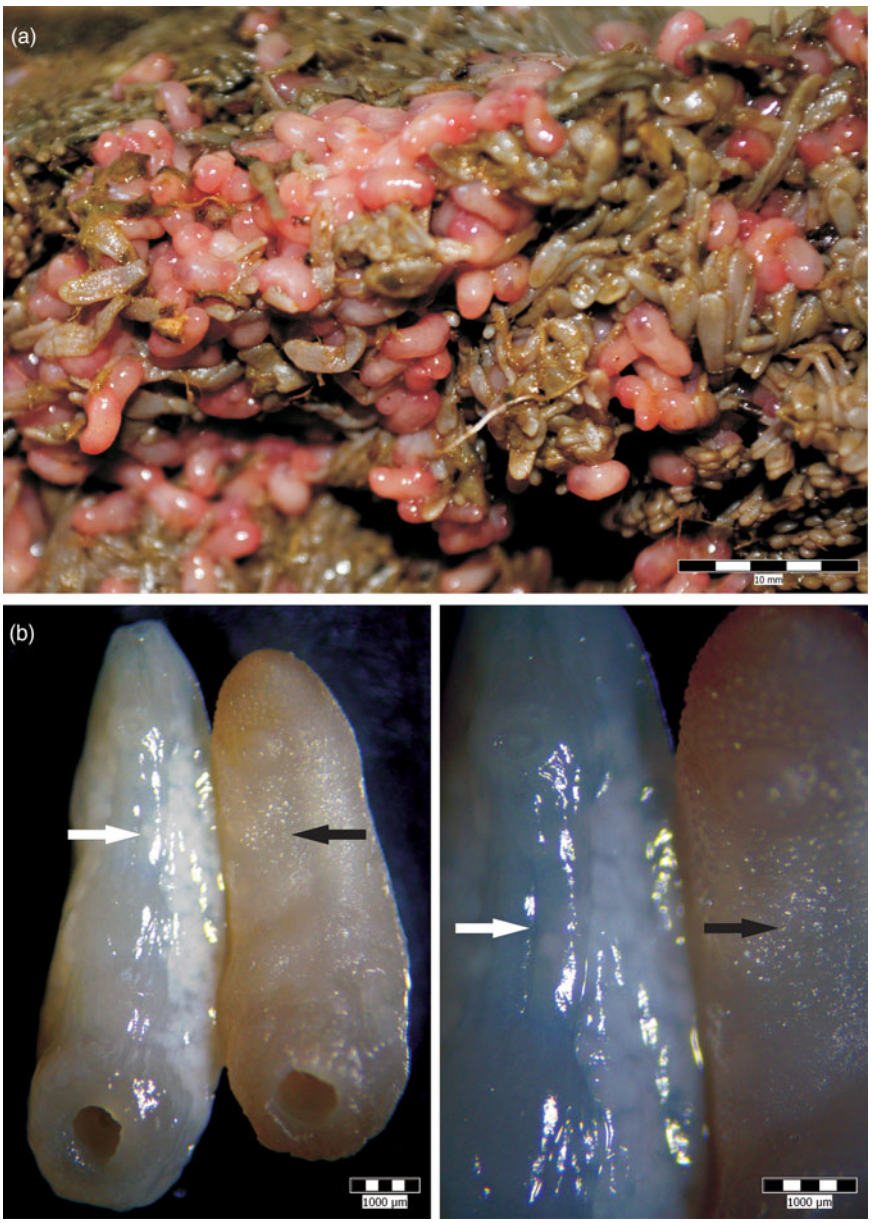

Fig. 1. (a) Rumen of a red deer heavily infected with adult flukes of *Paramphistomum leydeni* and *P. cervi*. (b) The tegument of both species at two magnifications to show the presence of papillae (black arrows) in *P. leydeni* and the absence of papillae (white arrows) in *P. cervi*.

features; for example, *P. cephalophi* has a posterior notch in the acetabular region, while *Gigantocotyle* spp. have an enormous acetabulum.

Morphological identification of flukes in our study depended on complete drying of the tegument, since moisture on the tegument surface can mask small tegumental papillae due to light reflection. This gives the false impression that the tegument surface is smooth and not rough, similar to other ruminal fluke species in Europe. Accurate morphological differentiation between *P. cervi* and *P. leydeni* also requires taking into account that the tegumental papillae of immature *P. leydeni* are visible only with the aid of scanning electron microscopy (Nikander & Saari, 2007). Failing to consider this possibility may lead to the false conclusion that papillae are absent and that the flukes are *P. cervi*. These considerations mean that previous reports of paramphistomosis should be interpreted with caution, since many authors did not report attempts to differentiate these two species. Indeed, Nikander & Saari (2007) concluded that rumen flukes in reindeer (*Rangifer tarandus*) in Finland were *P. leydeni*, rather than *P. cervi* as usually reported. In our case, the

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Table 1. Polymorphic sites identified in 320-bp sequences amplified from *Paramphistomum leydeni* and *P. cervi* DNA, and potential matches with GenBank sequences.

Sequence	Polymorphic site									GenBank matches
	64	183	195	206	234	250	258	259	287	
P. leydeni KX274232	C	T	C	A	T	T	C	G	C	KP341666, KJ995524, KJ995525, KJ995526, KJ995527, KJ995528, KJ995529, KJ995530, KJ995531, HM209064, AB973398
P. cervi KX274233	T	G	T	G	C	C	T	A	T	KJ459935, KJ459936, HM026462

presence of eggs in the fluke samples and faeces demonstrates fluke maturity and reduces the probability of misdiagnosing *P. leydeni* as *P. cervi* based on morphology.

Given the possibly worldwide distribution of paramphistomid flukes, it appears that paramphistomosis and its economic impact may be greater than previously thought (Lotfy *et al.*, 2010), especially in wildlife. While paramphistomosis is frequently studied in domestic animals, it is less studied in wild ruminants. Few details about the presence and identity of paramphistomes in cervids are known (O'Toole *et al.*, 2014), despite sweeping statements in the literature.

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Conflict of interest

None.

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