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First report of larval stages of *Fasciola hepatica* in a wild population of *Pseudosuccinea columella* from Cuba and the Caribbean

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Abstract

A wild population of the lymnaeid snail *Pseudosuccinea columella* infected by larval stages of *Fasciola hepatica* was discovered in the Pinar del Río Province, Cuba. One of 100 snails was infected in a rice culture field. This is the first time this species has been found acting as intermediate host of *F. hepatica* under natural conditions, not only for Cuba but also for the Caribbean area.

Introduction

Fasciolosis is a parasitic disease that affects humans and other mammals, especially ruminants, representing a medical and veterinary problem with a serious impact on public health and economy at different levels, from local to national level in several countries (Crossland *et al.*, 1977; Rangel & Martinez, 1994; Roberts & Suhardono, 1996; Claxton *et al.*, 1997; Mas-Coma *et al.*, 1999; Ortiz *et al.*, 2000). The parasite responsible for this disease is the cosmopolitan digenean trematode *Fasciola hepatica*, with a worldwide distribution, especially in regions where cattle-raising has been developed. The life cycle of *F. hepatica* includes the transmission through a freshwater snail intermediate host belonging to the Lymnaeidae family (see review in Hurtrez-Boussès *et al.*, 2001). In Cuba, only two lymnaeid species occur, *Fossaria cubensis* and *Pseudosuccinea columella*, but until now the former had been the only species found to be naturally infected in the field by the parasite (Melcon & Perera, 1994; Gutiérrez *et al.*, 2003a, 2005a, b). Although no report on *P. columella* harbouring parasite larvae under natural conditions had previously been made for the Caribbean area, this snail is also considered an effective host of *F. hepatica* as it acts as a natural intermediate host in countries such as Brazil, Australia and Argentina (Ueta, 1980; Boray *et al.*, 1985; Oliveira *et al.*, 2002; Prepelitchi *et al.*, 2003) and it shows a high susceptibility to miracidial exposures under laboratory conditions (Léon-Dancel, 1970; Boray *et al.*, 1985; Gutiérrez *et al.*, 2002).

In Cuba, both species occur at high densities in a variety of freshwater bodies, but *F. cubensis* shows a broader distribution throughout the island compared to *P. columella*, which is found from Pinar del Río through Camagüey provinces and seems to be absent from the eastern provinces Las Tunas through Guantánamo (Rojas *et al.*, 2010). Also, recent studies showed that, at least in Cuba, *F. cubensis* occupies the most anthropic habitats and *P. columella* is found more frequently in rural sites

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(Cañete *et al.*, 2004). Recently, we detected very high densities of *P. columella* in a rice culture area at the El Pilón locality, Pinar del Río province, and searched to see if some of these snails were naturally infected with intramolluscan stages of the parasite, since a number of cows were normally seen grazing in the area and cases of bovine and caprine fasciolosis had been reported in the neighbouring village of San Andrés. In this paper we report the first infection observed in naturally occurring *P. columella* from Cuba.

Materials and methods

Snail sampling and examination

The locality of El Pilón is situated west of Bahía Honda village in the municipality of the same name (22°50′45″N; 83°21′32″W). This area belongs to the mountain system Sierra de los Organos in the Pinar del Río province. The sampling site consisted of a field sown with rice, completely flooded over an area of nearly 300 m^2. The depth of water did not exceed 0.5 m, with a bottom mainly of mud and plant sediments. Suitability for the development of snail populations is granted for most of the year, with food from aquatic vegetation and water. As cattle-raising occurs in many farms in these localities, the life cycle of *F. hepatica* may be ensured.

Snail samples were taken on 17 December 2007 along the shores of the rice field. Snails were hand-collected from the mud or at the water surface using forceps. Because the abundance of their populations was extremely high, only 100 snails were randomly collected and saved in plastic boxes for laboratory studies. The presence of trematode infection was detected first by stimulation of cercarial shedding using a light source, and, second, by visual inspection of living snails for recognition of intramolluscan parasite development using a stereoscope. The search for infection in living snails lasted for a period of 15 days following snail collection. After this period the remaining snails were crushed and soft parts were analysed carefully, searching for parasite larvae. Each snail found harbouring trematode larvae was scored as infected. The hepato-pancreas of each infected snail was dissected to remove redial stages of trematodes, which were stocked at 80% ethanol for later molecular analysis.

Molecular identification of F. hepatica *intramolluscan stages*

DNA extractions of parasite larvae were performed using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In order to identify *F. hepatica* we amplified the rDNA gene, ITS-1 using the following primers, Lim1657 (forward): 5′-CTGCCCTTTGTACACACCG; and ITS1-RIXO: 5′-TGG-CTGCGTTCTTCATCG (Almeyda-Artigas *et al.*, 2000). For the amplification we used 2 μl of DNA template in a 25 μl reaction volume, containing: 5 μl each of polymerase chain reaction (PCR) buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 10 pmol of each primer and 1 U GoTaq DNA polymerase (Promega, Madison, Wisconsin, USA). Temperature cycling was: 2 min at 94°C, followed by 30 cycles for 30 s at 94°C, 50°C for 30 s, 72°C for 30 s and finally 7 min at 72°C.

The amplified products (5 μl) were verified on 1% agarose gels in Tris–acetate–EDTA buffer. DNA sequencing was performed by CoGenics Genome Express (Meylan, France) using PCR-amplified products as templates.

The obtained sequences were aligned using CLUSTAL-W implemented in BioEdit v.7.0.9 (Hall & BioEdit, 1999). We used BLASTN 2.2.22 (available at http://www.ncbi.nlm.nih.gov/BLAST) to detect homologue sequences.

Results

Three of the 100 snails sampled were found to be infected by redia of trematodes. The complete ITS-1 sequence obtained from trematode larvae found in *P. columella* from Cuba was 600 pb long. After BLAST analysis, our sequence was compared with some rDNA homologue sequences of *F. hepatica* available at http://www.ncbi.nlm.nih.gov (AB207139, AB207140, AB207141, AB385611, AJ243016). One of the three larval parasites showed no nucleotide difference with these five sequences. This similarity proves that the redia found in one *P. columella* snail from Cuba was *F. hepatica*. As reported previously, ITS-1 remains an appropriate marker for identification of this trematode species (Mera y Sierra *et al.*, 2009).

Discussion

In spite of being very susceptible to parasite infection, as shown in previous experimental exposures (Gutiérrez *et al.*, 2002), so far no specimen of *P. columella* had been found harbouring intramolluscan larvae of *F. hepatica* in the field in Cuba. El Pilón is the first Cuban locality where the presence of *P. columella* naturally infected by *F. hepatica* has been demonstrated. All snails collected at the El Pilón site, where infection by the parasite was found, had mantles showing the type of pigmentation considered to be a good marker of susceptible populations of *P. columella* to *F. hepatica* infection (see Gutiérrez *et al.*, 2003b).

The Caribbean area is a region where *F. cubensis* seemed to be the unique intermediate host of *F. hepatica*. This situation is probably due to the fact that this snail is known as a local species, probably occurring in this area for a long time. It was apparently already distributed all over Cuba when it was first described by L. Pfeiffer at the beginning of the 19th century (Pointier *et al.*, 2005). On the contrary, *P. columella* originates from North America and is known as an invasive species which presently has a worldwide distribution (Pointier, 2008). In Cuba *P. columella* was reported for the first time by A. Poey in the middle of the 19th century, but at the present time the species only occurs from Pinar del Río to Camagüey provinces (Rojas *et al.*, 2010). In the Caribbean area *P. columella* was reported from Puerto Rico (Van der Schalie, 1948), Dominican Republic (Gómez *et al.*, 1986), Venezuela (Malek & Chrosciechowski, 1964) and Guadeloupe (Pointier, 2008) but was never recorded as an active intermediate host of *F. hepatica* in the field. However, as already pointed out, in several other invaded countries, such as Australia, Brazil and Argentina, *P. columella* has been reported to be naturally infected by *F. hepatica* (Ueta, 1980; Boray *et al.*, 1985; Prepelitchi *et al.*, 2003). Consequently, the discovery of naturally infected

P. columella in Cuba shows that this species must be seriously taken into account in the development of future control programmes of this parasitosis.

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