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Fasciola hepatica in wild boar (*Sus scrofa*) from Italy

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ABSTRACT

Fasciola hepatica is a trematode infecting ruminants worldwide, occasionally reported in a wide range of animal species, including humans. According to the WHO, fasciolosis is recognized as a re-emerging neglected tropical disease, responsible for endemic and epidemic outbreaks in humans. Although the main hosts of the parasite are represented by cattle, sheep and goats, wildlife may be involved in its circulation. Here we firstly report *F. hepatica* in a wild boar from Italy (southern area) and characterize it both morphologically and molecularly. The *nad*1 gene analysis of specimens analyzed, revealed a high genetic similarity with those of humans from Iran and Peru, as well as a close phylogenetic relationship to those in ruminants from Brazil, Ecuador and Egypt. Considering the increase in the wild boar populations in urban and peri-urban areas, a potential role of this ungulate in the circulation of this zoonotic trematode is suggested.

1. Introduction

Fasciolosis caused by *Fasciola hepatica* and *Fasciola gigantica* (Trematoda: Fasciolidae) is a foodborne parasitic disease with global distribution, affecting a wide range of domestic and wild mammalian species, both herbivorous and omnivorous [1].

The two main species within the genus have a different epidemiology, being *F. hepatica* worldwide distributed and *F. gigantica* confined to some areas of Africa and Asia [2]. The adults of *F. hepatica* are localized in the biliary ducts of herbivores (main definitive hosts) whereas humans may be infected through the accidental ingestion of water and plants (e.g., watercress) carrying metacercariae [3]. Briefly, un-embryonated eggs pass into the stool of the definitive host, become embryonated in freshwater and release miracidia which invade snails (i.e., the intermediate host of the Family Lymnaeidae) becoming cercariae which are released in the freshwater [4]. On the marsh vegetation cercariae become infective metacercariae, which may be ingested by animals through raw vegetables and contaminated water, especially in grazing areas [5]. Depending on the load of ingested metacercariae, the disease can lead to a traumatic hepatitis or chronic hyperplastic cholangitis [6,7]. Globally, human fasciolosis is considered a re-emerging disease [1,8] affecting between 2.4 and 17 million people, in more than 70 countries, in which climatic and environmental changes may potentially impact on the parasite spreading [2,9]. In tropical countries, fasciolosis often reaches high infection rates in farm animals [10,11], causing over 3 billion dollars of annual productivity loss in the global livestock industry [1,4]. Infections by *F. hepatica* are commonly associated to ruminants [12] and less frequently to pigs since they usually do not feed in contaminated pastures [13]. However, in the last decades, several cases of *F. hepatica* adults were reported in wild animals from different European countries, such as red deer (*Cervus elaphus*) from Scotland [14], roe deer (*Capreolus capreolus*) from Spain [15], fallow deer (*Dama dama*) from Italy [16], elks (*Alces alces*), otters (*Lutra lutra*) and European beavers (*Castor fiber*) from Belarus [17], European rabbits (*Oryctolagus cuniculus*) from France [18], hares (*Lepus europaeus*) from the Netherlands [19], nutria (*Myocastor coypus*) from France [20], and wild boars (*Sus scrofa*) from Spain [21]. The high prevalence (11.2%) of fasciolosis in wild boars from Spain suggested that this ungulate was the secondary reservoir of the trematode, after cattle and small ruminants [21]. In the last decades, wild boar populations have increased throughout many countries in Europe, such as in Italy [22], invading agricultural areas, pastures and water sources [23,24]. Considering the intensive farming of ruminants in many countries, wild boars may favor the maintenance of the biological life cycle of the parasite in the

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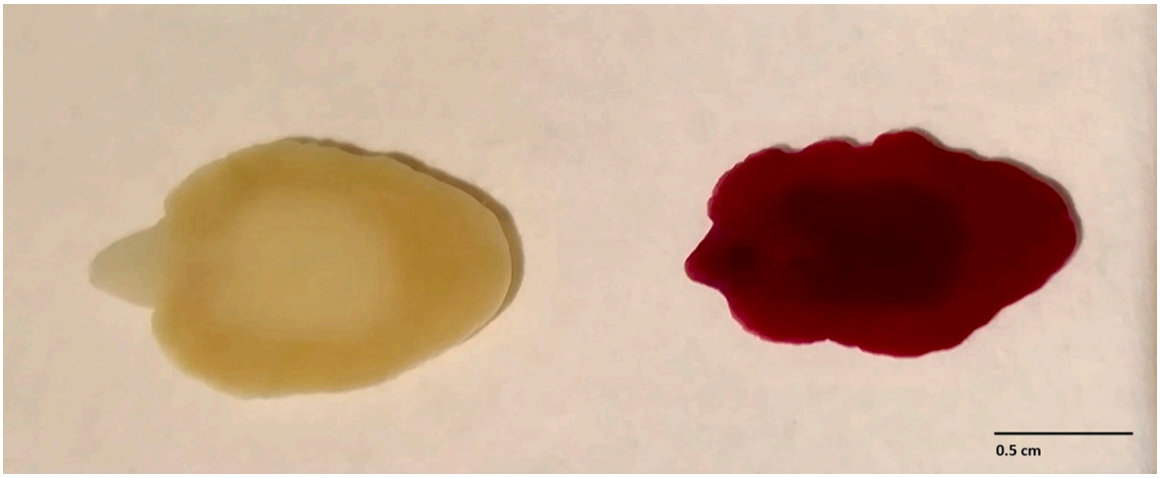

Fig. 1. Macroscopic observation of *F. hepatica* adult specimens retrieved from a wild boar liver, without (left) and with (right) the Grenacher's Borax Carmine acid staining.

environment. However, data on the occurrence of *F. hepatica* in wildlife in Europe are limited to incidental observations [14] and the epidemiological role of these ungulates in the maintenance and transmission pathways is still under discussion. Herein, we report *F. hepatica* infection in a wild boar from Italy and discuss the potential role of this animal species in the epidemiology of the disease.

2. Materials and methods

In October 2020, a two years old female wild boar was culled according to a regional monitoring plan for the control of zoonosis in wildlife (authorization n° "Decreto Dirigenziale 210-Piano B7 DPAR 2018") by regular hunters within an official hunting area of southern Italy (Salerno province - 40.343503 °N, 15.129785 °E). The animal was aged by using the examination of the teeth [25] and a complete necropsy was performed by an official veterinarian of the local health service. During the routine examination for metacestodosis, trematodes were found in the liver bile ducts after the incision at the base of the caudate lobe. Helminths were collected, stored in 70% ethanol and delivered to the Department of Veterinary Medicine, University of Bari Aldo Moro (Italy) for morphological and molecular analyses.

2.1. Morphological analysis

Helminths collected were stained with the Grenacher's Borax Carmine acid staining using a modified procedure as described [26]. Briefly, one specimen was previously washed with a fixing solution (i.e., 5 parts glacial acetic acid, 10 parts of 10% formalin, 85 parts of 85% ethanol), successively soaked in the Borax Carmine dye (1 h) and in water (4 h) to remove the excess of dye. A morphometrical examination of the helminths body size was performed using an optical microscopy (Leica DM-LB2) and all measurements obtained with Leica Las version 4.5.0 software (Leica Microsystems, Wetzlar, Germany) were compared to those available in literature [21]. Flukes were then dissected for the egg observation.

2.2. DNA extraction, PCR and sequencing

For molecular identification, DNA was extracted from all isolated specimens using a commercial kit (DNeasy® Blood & Tissue kit, Qiagen, Hilden, Germany), according to the manufacturer's instructions. A fragment of NADH dehydrogenase subunit 1 (*nad*1) mitochondrial gene (535bp in size) was amplified by the primers Ita 2 (5'- GGAGTACGGTTACATTCACA -3') and Ita 10 (5'- AAGGATGTTGCTTTGTCGTGG -3') [27]. The PCR run protocol was modified as follows: initial denaturation at 95 °C for 10 min, followed by 30 cycles at 94 °C for 90 s, 55 °C for 90 s, and 72 °C for 120 s, with a final extension at 72 °C for 7 min. All PCR products were examined on 2% agarose gels stained with GelRed (VWR International PBI, Milan, Italy) and visualised on a GelLogic 100 gel documentation system (Kodak, New York, USA). Amplicons were then purified and sequenced in both directions using the same primers as for PCRs, by the Big Dye Terminator v.3.1 chemistry in a 3130 Genetic Analyzer (Applied Bio-systems, Foster City, CA, USA). Sequences were edited and analysed by the Geneious software version 9.0 (Biomatters Ltd., Auckland, New Zealand) [28] and compared with those available in the GenBank database by the Basic Local Alignment Search Tool (BLAST; blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1
Morphological parameters evaluated for the *F. hepatica* specimens (F1, F2, F3) retrieved from a wild boar liver, including minimum and maximum measurements and average size. All measurements are reported in millimetres (mm), excepted for the Body Area (square millimetres - mm^2).

Measurements (mm)	F1	F2	F3	min	max	average
Body Length	16.9	14.2	14.0	14.0	16.9	15.0
Body Width	9.2	8.3	7.8	7.8	9.2	8.4
Body Perimeter	38.0	33.5	33.9	33.5	38.0	35.1
Body Area (BA) (mm^2)	92.5	86.4	83.2	83.2	92.5	87.4
Cone Length	2.4	2.1	2.1	2.1	2.4	2.2
Cone Width	2.5	2.3	2.2	2.2	2.5	2.3
Testicles Length	7.3	6.8	7.2	6.8	7.2	7.1
Testicles Width	4.7	3.9	4.5	3.9	4.7	4.4
Ovary Width	6.7	5.5	6.5	5.5	6.7	6.2
Diameter Ventral Sucker	1.0	0.7	0.8	0.7	1.0	0.8
Diameter Oral Sucker	0.9	0.9	0.9	0.9	0.9	0.9
Distance Ventral Sucker - posterior body	12.9	11.3	10.2	10.2	12.9	11.5
Distance Oral Sucker - Ventral Sucker	1.4	1.3	1.2	1.2	1.4	1.3
BL/VS-P ratio	1.3	1.3	1.4	1.3	1.4	1.3

2.3. Phylogenetic analysis

The phylogenetic analysis was based on 499bp of the *nad*1 gene sequence of *F. hepatica* detected from humans and several animal species, including available sequences from the GenBank database. Phylogenetic relationship was inferred by the Maximum Likelihood (ML) method based on Tamura's 3-parameter model [29] and Gamma distribution used to model evolutionary rate differences among sites (+G) selected by best-fit model [30]. Evolutionary analyses were conducted on 8000 bootstrap replications using the MEGA X software [31].

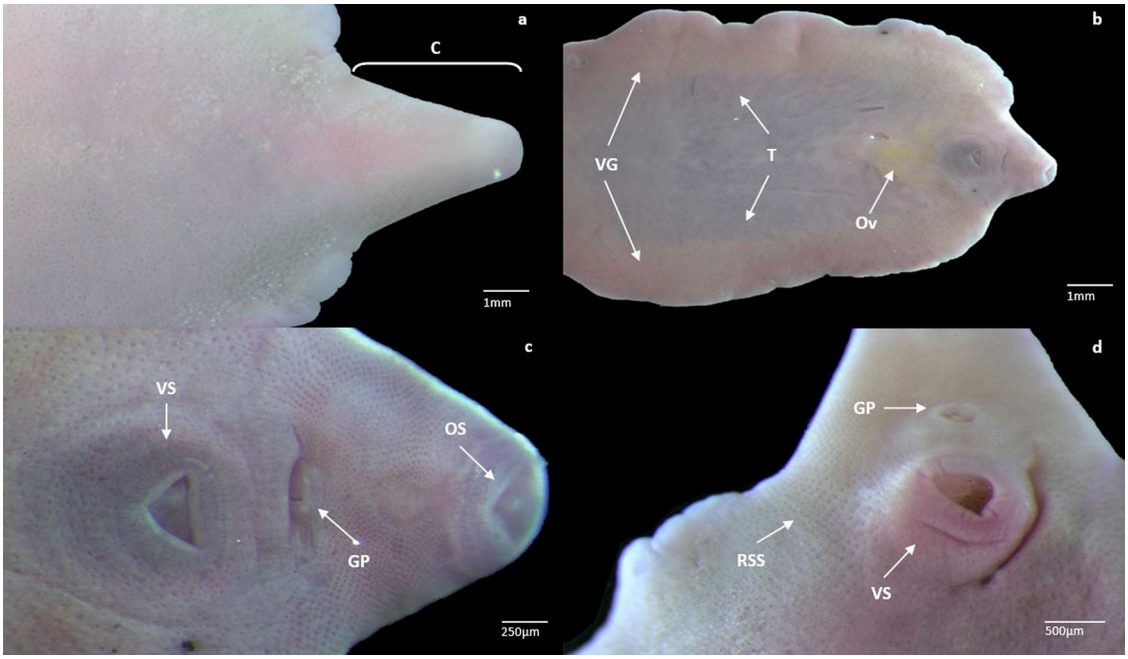

Fig. 2. Microscopic observation of *F. hepatica* specimens stained with Grenacher's Borax Carmine acid: (a) dorsal side showing the Cone (C) structure; (b) ventral side with visible Ovary (Ov), Testicles (T) and Vitelline Glands (VG); (c) anterior surface showing ventral sucker (VS), oral sucker (OS) and Genital Pore (GP); (d) reverse scaly spines (RSS) on the Cone surface, ventral sucker (VS) and Genital Pore (GP).

Homologous sequence of *F. gigantica* in cattle (*Bos taurus*) from Vietnam was used as outgroup (accession number: AB385618).

3. Results

Helminths (n = 3) collected from the bile ducts of the wild boar liver during necropsy were identified as adults of *F. hepatica* by the morphological and morphometric analysis. The difference between the parasitic specimens with and without Grenacher's Borax acid staining is shown in Fig. 1. Data regarding the body size measurements and morphological details of *F. hepatica* are shown in Table 1 and Fig. 2. Operculated eggs were observed in the uterus measuring 128.6 µm × 69.6 µm with thin ellipsoidal shell. Molecular analysis of the *nad*1 partial gene sequences confirmed the identification of *F. hepatica*, displaying a 100% nucleotide identity compared to the sequences available in the GenBank database. The phylogenetic tree (Fig. 3), based on the *nad*1 sequences of *F. hepatica* herein detected, revealed that these latter clustered together with those retrieved in farm animals from different countries (i.e., sheep from Egypt - LC076259; cattle from Brazil - MK838754, Ecuador - LC273181 and Egypt - LC273166). Sequences herein obtained from the *nad*1 gene of *F. hepatica* were deposited in GenBank under the accession number (MW995947) (http://www.ncbi.nlm.nih.gov). No pathological alterations of the hepatic serosa or thickening of the bile ducts were observed at the necropsy.

4. Discussion

The description of *F. hepatica* in a wild boar from Italy confirms these ungulates as suitable hosts of this zoonotic trematode, as previously reported in Spain [21], United Kingdom [32] and Serbia [33].

The absence of any pathological alterations in the wild boar liver herein examined may be due to the low number of adult mature flukes retrieved during necropsy. Conversely, in highly infested wild boars, white dots or white button-like cysts were detected on the liver surface [32] along with migration tracks and cholangitis [7]. Overall, the severity of pathological manifestations depends on the number of adults [6]. *Fasciola hepatica* specimens herein measured were similar to those of the liver fluke population in wild boars from Spain [21], suggesting a low variability in size and shape of the parasites among these ungulates from different European countries. In addition, the measurements observed in this study confirm that *F. hepatica* from wild boars has an intermediate size between those collected in sheep and cattle [21], therefore suggesting a strong relationship between this animal species and phenotypic features of adult parasites [34]. The presence of eggs in the uterus of the trematode demonstrated that this host may be involved in the spreading of this parasite.

The high nucleotide identity among sequences of *F. hepatica* from wild boar and those reported in ruminants (e.g., cattle from Brazil, Ecuador, Egypt and sheep from Egypt) and humans from Iran and Peru, highlights the importance of wild boar in maintaining the infection in a given area. This is also confirmed by the high infection prevalence (i.e., 40.0%) recorded in the faeces of wild boar from Spain [21] and Serbia (i.e., 5.5%) [33]. In addition, the decrease in extensive farming in Europe, including Italy [35,36] and the exponential increase of wild boar populations in rural and peri-urban areas [22,37] may enhance the importance of wild boars in the distribution of *F. hepatica*. A single study reports an infection prevalence of fasciolosis in 4.4% of feral Nebrodi black pigs in Italy (i.e., Sicily region in southern Italy) [13], therefore large-scale surveys should be run to investigate the existence of a sylvatic life cycle of the parasite. The role of wild boar in the maintenance of this trematode's species is also supported by the close genetic relationship of the specimens herein detected and those infecting humans and ruminants, therefore suggesting the public health concern represented by the boar populations in many urban and peri-urban areas of Europe [22,38].

In this scenario, a close cooperation between clinicians and veterinarians should be established for mitigating social issues related to the wild boar density and abundance (e.g., road accidents, agricultural crop damages), but also for preventing and minimizing the spread of many zoonotic parasites, including *F. hepatica*.

Declaration of Competing Interest

The authors declare that they have no known competing financial

Fig. 3. Phylogenetic relationship of *F. hepatica* detected in wild boar in this study with those available in literature from humans and farm animals. The phylogenetic analysis was based on 499bp *nad*1 gene sequences and inferred by the Maximum Likelihood (ML) method based on Tamura 3-parameter model and Gamma distribution used to model evolutionary rate differences among sites (+G) selected by best-fit model. Evolutionary analyses were conducted on 8000 bootstrap replications using the MEGA X software. Homologous sequence from *Fasciola gigantica* was used as outgroup (accession number: AB385618).

interests or personal relationships that could have appeared to influence the work reported in this paper.

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