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Original papers

Molecular characterization and seroprevalence of *Echinococcus granulosus* in wild boars (*Sus scrofa*) in south-western Iran

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ABSTRACT. This study presents the first molecular and serological evaluation of *Echinococcus granulosus* infections in wild boars in Iran. Twenty five wild boars were collected in south-western Iran, during authorized hunting program, from March to October 2013, necropsied and examined for *E. granulosus* infection. Furthermore, seroprevalence of cystic echinococcosis in hunted boars was evaluated by an ELISA system. A fertile hydatid cyst due to *E. granulosus* was detected in the lung of one of the animals. Genotype analysis of the isolate was determined by analyzing a mitochondrial gene, cytochrome C oxidase subunit 1 (*co1*). DNA was extracted from the cyst sample and polymerase chain reaction amplification and DNA sequencing of the specific region of the *co1* gene was performed. Molecular evaluation confirmed the presence of a sheep strain, the G1 genotype, in the wild boar in south-western Iran. This is the first report of the presence of G1 genotype of *E. granulosus* in wild boar in Iran. Serological evaluation of hydatid cyst by antigen-B ELISA revealed *E. granulosus* antibodies in 5 (20%) of 25 wild boars. A statistically significant difference was observed between the prevalence of *E. granulosus* antibodies and gender while the difference between the seroprevalence of *E. granulosus* and age was insignificant. Findings of this study might have important implications for the prevention and control of cystic echinococcosis.

Key words: *Echinococcus*, seroprevalence, genotype, wild boar, Iran

Introduction

Cystic echinococcosis (CE) is a parasitic disease of domestic animals and humans caused by the larval stage of tapeworm *E. granulosus* sensu stricto [1]. Humans are infected by accidental ingestion of worm eggs through contaminated food, water or soil [1]. CE in animals and humans is a significant economic and public health problem in the Middle East countries, largely Iran, Iraq and Turkey [2–3]. Wild boars are an intermediate host of *E. granulosus* and might play a significant role in sylvatic cycle of CE. Infections of wild boars with *E. granulosus* have been reported from different areas of the world [4–9]. Wild boars, is a widespread animal all over the tropical Asia. Geographical distribution of wild boar in Iran is seen in north dense rainforests, north-west and west oak forests, and south-west tropical forest [10]. Wild boars are omnivorous animals and feed on plants (leaves, grasses, fruits; roots and bulbs) and animals (mice, birds and small amphibious) but yet eat another creatures such as insects, insect larvae, beetles and reptiles.

Infection of wild boars with *E. granulosus* larvae in Iran has been previously documented by Eslami and Farsad–Hamdi where they found that 5% of wild boars collected from the north, northeast, and southwest of Iran are infected by *E. granulosus* larvae [4]. However, no molecular evaluation had been performed in their study and the genotype of

the parasite was remained indeterminate. Up to now, two strains of *E. granulosus* sensu lato have been identified in wild boars, mainly G1 and G7 [6–9,11–12]. These two strains have different life cycles and also different genetic and biochemical characteristics [1]. The current study aimed to assess the seroprevalence of CE in wild boars in south-western Iran and also to determine the genotype of *E. granulosus* in these animals.

Materials and Methods

Study area. This study was done in Bushehr province (28.9184ºN, 50.8382ºE), located in south-western Iran. Unique environmental feature of this province, with highland nature and dense forests in the northern part, makes this area a suitable habitat for wild boars and other wild animals.

Collecting blood and tissue sample. After getting approval from the ethics committee of Shiraz University of Medical Sciences, with coordination to local hunters, twenty five adult wild boars, including 14 females and 11 males, were collected during authorized hunting program, from March up to October 2013. Approximate age (based on teeth development) and gender of the boars were recorded. Immediately after the hunting, blood samples were collected from each boar. Sera were collected from the blood samples and stored at -20°C until use. At necroscopy, all parts of tissues of the boars were carefully checked for presence of any type of cysts. Fluid of any fluid-filled cysts were aspirated and centrifuged at 1000g for 5 minutes and the sediment was microscopically examined. Cyst-containing tissues were cut and fixed in 10% buffered formalin for histopathological evaluation. Tissue sections of the cysts were prepared and stained with hematoxylin and eosin (H&E) stains and studied under light microscope. Furthermore, a part of cyst and also isolated protoscolices were kept in alcohol, for molecular studies.

DNA extraction and molecular evaluation. For molecular evaluation, DNA was extracted from both germinal layer and protoscolices, using genomic DNA extraction kit (QIAamp DNA Mini Kit, QIAGEN GmbH, Germany) based on the manufacturer's instructions. Polymerase chain reaction (PCR) was performed on a mitochondrial gene, cytochrome C oxidase subunit 1 (*co1*). A 480 bp fragment of *co1* gene was amplified, using JB3, TTTTTTGGGCATCCTGAGGTTTAT, and JB4, TAAAGAAAGAACATAATGAAAATG, primers [13].

For PCR amplification, 50 µl of reaction volumes including 5 µl of extracted DNA, 10 pmol of each primer, 1.5 mM of $MgCl_2$, 1.25 units of Taq DNA polymerase, 0.2 mM of dNTP and 10x PCR buffer were prepared. PCR was carried out, using one cycle of initial denaturation at 95ºC for 5 min, followed by 40 cycles of denaturation at 94ºC for 1 min, annealing temperature at 55ºC for 1 min, extension temperature at 72ºC for 90s, and final extension at 72ºC for 5 min. PCR product of *co1* gene was purified from the agarose gel, using Vivantis DNA purification kit (Vivantis Technologies Sdn. Bhd. Selangor Darul Ehsan, Malaysia) and sequenced, applied the same forward and reverse primers used for amplification. For strain identification, the sequence was aligned, using CLCS QIAGENE software, and compared with those of existing *co1* reference sequences related to the genotypes of *E. granulosus* in the GenBank.

Serological evaluation. Blood samples collected from the wild boars were assessed for antibodies to *E. granulosus* by an Enzyme Linked Immunosorbent Assay (ELISA). Antigen B, prepared from sheep hydatid cyst fluid, was used for the ELISA. Antigen B is the most sensitive and specific antigen for the serodiagnosis of CE [14–16]. ELISA was performed in flat-bottom 96-well microplates (Nunc, Denmark). Microplate was coated with 5 µg/ml of antigen B (100 µl/well) in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) by overnight incubation at 4°C. Excess antigen was removed by washing the plate in phosphate buffered saline-Tween 20 (PBST, pH 7.4 containing 0.05% Tween 20). Unbound sites were blocked by 3% (in PBST) skimmed milk. Following washing as before, different dilution of wild boar serum samples (100µl of 1/50 and 1/100 dilutions in PBST) were added to the wells of microplates and incubated at room temperature for 1.5 hour. After washing, horseradish peroxidase (HRPO) conjugated anti-swine antibodies (Sigma, SAB 3700427) at 1/1000 dilution in PBST was applied to the plate and incubated at room temperature for 1 hour. Following washing, 100 µl/well of chromogen /substrate (0.4 mg/ml OPD, 0.025% H_2O_2 in 0.1 M citrate buffer, pH 5) was added to the plates and the absorbance values of the wells, at 490 nm, were determined by a microplate reader, after 30 minutes.

Results

A fluid-filled, hydatid cyst with dimensions between 40×60 mm was found in the lung of a male wild boar (Fig. 1A). The fluid-containing cyst was fully fertile, with numerous evaginated and invaginated protoscolices (Fig. 1B-C). Hematoxylin and Eosin stained sections of the cyst showed typical hydatid cyst layers (Fig. 1D).

DNA was extracted from protoscolices and also the germinal layer of the cyst and PCR-amplified. The PCR result revealed a 480 bp fragment of *E. granulosus co1* gene (Fig. 2). To find out the strain of *E. granulosus*, the sequence of the *co1* of the isolate was aligned and compared with those *co1* sequences of *E. granulosus* available in the GenBank. The sequence of the isolate showed 100% identity with available sequences of *Echinococcus* G1 genotypes, including JQ250814.1 (from camel in Iran), KM100575.1 (from sheep in Turkey), AB688600 (from sheep in Jordan), and AB688139.1 (from human in Altai region of Russia).

Serological evaluation of hydatid cyst by AgB-ELISA revealed *E. granulosus* antibodies in 5 (20%) of 25 wild boars. Statistically significant differences were observed between the prevalence of *E. granulosus* antibodies and gender where higher seroprevalence was observed in male wild boars. No statistically significant difference was observed between prevalence of *E. granulosus* antibodies and age.

Discussion

Molecular strain differentiation of *E. granulosus* sensu lato within areas endemic for CE is important for proper implementation of any control or interventional programs related to this zoonotic disease. Using molecular approaches, ten distinct genotypes (G1-G10) have been identified for *E. granulosus* sensu lato so far [17]. The sheep strains of *E. granulosus* sensu stricto (G1 genotype) is the most common strain documented in humans and animals worldwide, while the horse and pig strains

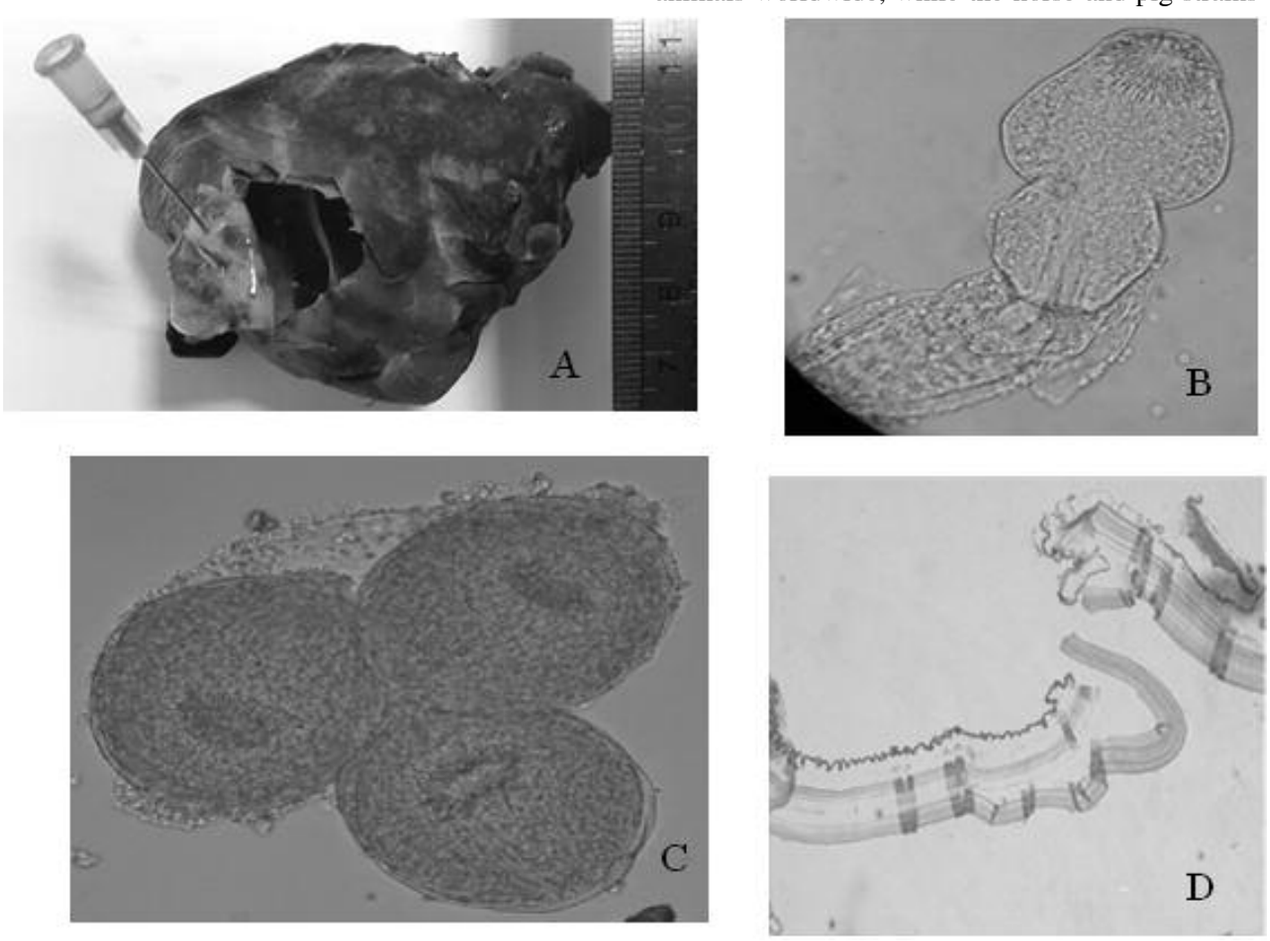

Fig. 1. Hydatid cyst of lung isolated from the wild boar. A. Hydatid cyst in lung, B. evaginated protoscolex of *E. granulosus*, C. invaginated protoscolices, D. pathological section of hydatid cyst shows the laminated and germinal layer of the cyst (×40, H & E).

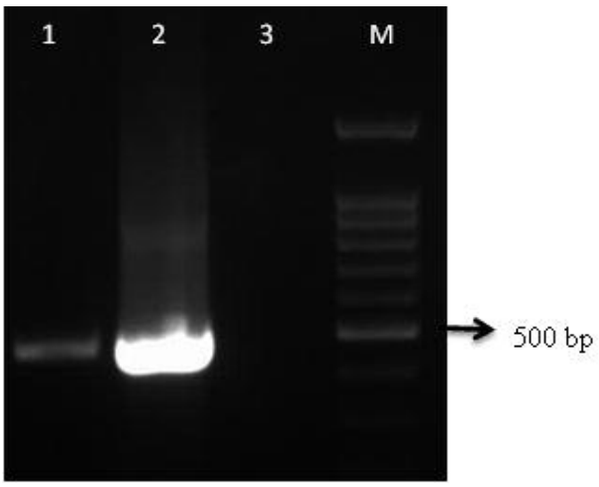

Fig. 2. Agarose gel electrophoresis of *co1* gene PCR product of *E. granulosus* recovered from the hydatid cyst in the lung of a wild boar from Iran.
1. DNA of the sample isolated from the lung of wild boar,
2. Positive control (DNA from sheep hydatid cyst),
3. Negative control, M (100 bp molecular marker).

(G7) may have no or low infectivity for human [17]. Similar to the findings in other areas of the world, epidemiological data show that the *E. granulosus* sensu stricto is the predominant strain in human cases of hydatid cyst in Iran [18]. The current study, for the first time, documented the presence of G1 strain of *E. granulosus* in wild boar in Iran. Infection of wild boars by *E. granulosus* larvae in Iran has been previously reported by Eslami and Farsad–Hamdi from the north, northeast, and southwest of the country [4]. However, the genotype of the parasite was not determined in their study. In another study in the region, Solaymani-Mohammadi evaluated the helminthic infections of 12 wild boars in Lurestan province, western Iran, but no case of *E. granulosus* larvae was found in their study [10]. Wild boars are likely to be involved in the epidemiology of CE by acting as intermediate hosts for *E. granulosus* in its sylvatic cycle. Previous reports documented that *E. granulosus* isolates from wild boar corresponded predominantly (µ75%) to the pig strain, G7 genotype [11,19]. Yet other genotypes of *E. granulosus*, including G1 and G6, have been reported in wild boars in various studies from different geographical areas of the world, which in turn indicates that wild boars can be infected by multiple genotypes of *E. granulosus* [6–9, 11–12]. Martin-Hernando et al. [6] reported a G1 strain of *E. granulosus* in wild boars in Spain and Sanchez et al. [8] reported the presence of the G1 along with the G7 genotypes in wild boars from Peru. Umhang et al. [11] described the pigs and also wild boar infections with G6/7 genotype of *E. canadensis* from Corsica, an island in the Mediterranean Sea. Onac et al. [9] reported a prevalence of 12.3% for hydatid cyst in wild boars in Romania. The strain of the parasite was G1 in 45.5% and G7 in 39.4% of cases. The isolated cyst in the wild boar in this study was fertile, with numerous protoscolices. This suggests a higher risk of transmission to other animals through consumption of wild boars carcasses. Similar observation was reported in Sánchez et al. [8] study where they found that all of the cysts in lung of the studied wild boars are fertile. In our study the hydatid cyst was in lung of the infected boar. Hydatid cysts of lung are more frequent in swine and wild boar although liver infection has also been reported in these animals [1,4,19]. Findings of the current study suggest that wild boar could be involved in the epidemiology of *E. granulosus* sensu stricto in the region, in view of that large amounts of carcass remains are available to dogs and other carnivores during the hunting season.

Serological studies revealed a relatively high prevalence of hydatid cyst in wild boars in this study. However, hydatid cysts were not detected in all of seropositive cases. The reasons for that might be that the cysts have been too small to be detected, have been in an unreachable place, overlooked during necroscopy or calcified.

Conclusions

The findings of the current study revealed that the rate of hydatid cysts in wild boars in south-western Iran is relatively high. The results also confirmed the presence of the sheep strain of *E. granulosus*, G1 genotype, in wild boars in Iran. These findings have important implications for the prevention and control of this zoonosis in the area.

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