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RESEARCH NOTE

First Detection and Molecular Characterization of *Echinococcus equinus* in a Mule in Turkey

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Abstract

Cystic echinococcosis is a zoonotic disease with a cosmopolital distribution. It is caused by the larval stages (metacestodes) of the parasite *Echinococcus granulosus* which infects different animal species. In this report, we present a case of *E. granulosus* infection in a mule and molecular characterization of the cyst. For this purpose parasite material was collected from the liver of a necropsied mule. DNA was isolated and PCR amplification of mitochondrial 12S rRNA as well as partial sequencing of mitochondrial cytochrome c oxidase subunit 1 (mt-CO1) genes were performed. Six unilocular cysts, filled with clear fluid were found in the liver and spleen. All cysts were found to be fertile. The 12S rRNA-PCR did not yield any band while mt-CO1-PCR yielded a 446 bp sized amplification product. Sequence corresponding to mt-CO1 gene was identical to a sequence reported for *E. equinus* (formerly G4) (Genbank accession number: KC953029). This is the first record of *E. equinus* as a cause of cystic echinococcosis in a mule in Turkey.

Keywords

Echinococcus equinus, mule, genotyping, PCR, Turkey

Cystic echinococcosis (CE) caused by the metacestode of the dog tapeworm, *Echinococcus granulosus*, is a cosmopolitan zoonotic disease which infects different animal species (Rausch, 1995). Based on the molecular genetic characterization of mitochondrial and nuclear genes of *E. granulosus*, ten different genotypes, among which G4 (horse strain) have been already characterized (Nakao *et al*., 2007; Saarma *et al*., 2009). Recent taxonomic classification suggested the presence of five valid species within the *E. granulosus*. These are *E. granulosus s.s.* (G1-G3 genotypes), *E. equinus* (G4 genotype), *E. ortleppi* (G5 genotype), *E. canadensis* (G6–G10 genotypes) and *E. felidis* (Nakao *et al*., 2010).

E. equinus was firstly described in Great Britain by Williams and Sweatman (1963). Having the classical morphology, the parasite can develop and mature in foxes (Howkins *et al*., 1965), and the prepatent period can extend to approximately 70 days (Cook, 1989). The intermediate hosts of *E. equinus* are equids (horses, donkeys, and zebras) (Jenkins *et al*., 2005). Other Echinococcus species may also cause CE in horses, however those cysts are not usually fertile (Varcasia *et al*., 2008). Canids (dogs and probably foxes) serve as definitive hosts of *E. equinus* (Thompson and McManus, 2002). *E. equinus* appears to be a specific parasite of equids. The hazard of equids' cystic echinococcosis on public health is negligible because *E. equinus* is non-pathogenic for human (Romig *et al*., 2006). In equids, CE is generally a rare finding, mostly incidentally diagnosed at slaughter or postmortem examination.

Cystic echinococcosis remains one of the most important helminthic diseases in Turkey. There are several reports about *E. granulosus* strains circulating in the country. *E. granulosus sensu stricto* (G1-G3 complex) and *E. canadensis* (G6 and G7 genotypes) have already been reported both in livestock and humans (Snabel *et al*., 2009; Simsek *et al*., 2011a; Simsek *et al*., 2011b). The aim of the present study is to provide a molecular characterization of the metacestode obtained from a mule in Turkey with PCR amplification of mt-12S rRNA and partial sequencing of the mt-CO1 genes and the pathological screening as well. A two-year-old male mule was euthanized due to femur fracture in Surgery Department in Firat Univer-

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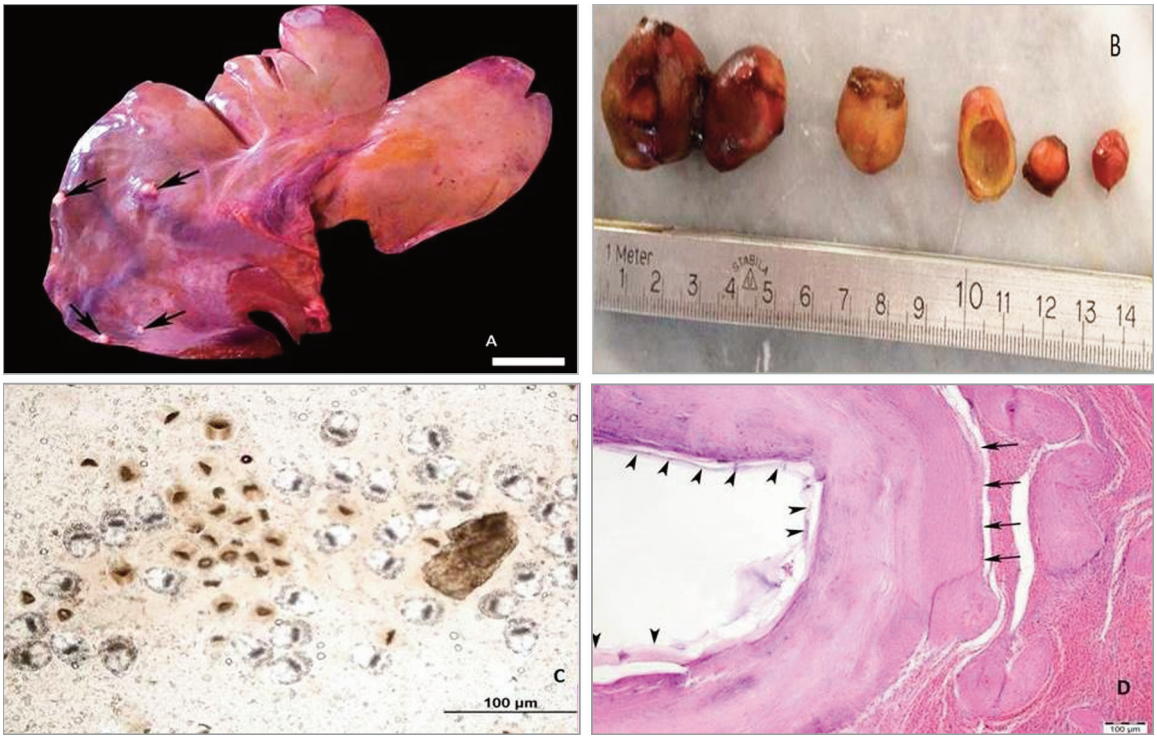
sity Faculty of Veterinary Medicine (Elazig-Turkey) and was referred to Pathology Department for necropsy. It had a poor body condition and insufficent abdominal adipose tissue. After necropsy, tissue speciments were collected from the visceral organs, brain and cysts. Tissues were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. The serial sections in 4–6 µm in thickness were stained with hematoxylin and eosin. For the purpose of molecular characterization, cyst material was collected from liver and fixed in 70% ethanol until use. Genomic DNA was extracted from both protoscoleces and cyst walls (germinal and/or laminar layers) using a commercially available DNA extraction kit (MBI Fermentas, Lithuania) following the manufacturer's instructions. Prior to extraction, cut tissues were washed with PBS for at least five times. E.g.ss1.for (5′-GTATTTTGTAAAGTT GTTCTA-3′) and E.g.ss1.rev (5′-CTAAATCACATCATCT TACAAT-3′) primer pair was used to amplify partial mt-12S rRNA of *E. granulosus* sensu stricto (Dinkel *et al.*, 2004). PCR reaction was carried out in a final volume of 50 µl, containing 5 µl 10X PCR buffer, 5 µl 25 mM $MgCl_2$, 250 µM each of dNTP, 20 pmol of each primer, 200 ng of template DNA, and 1.25 IU of TaqDNA polymerase (MBI Fermentas, Lithuania). The PCR products were separated on agarose gels (1.4%) and stained with ethidium bromide. Fragments of the CO1 mitochondrial gene were amplified as reported by Bowles *et al*. (1992) using the JB3/JB4.5 primers (5′-TTTTTTGGGC ATCCTGAGGTTTAT-3′/5′-TAAAGA AAGAACATAATGA-AAATG-3′). PCR amplification was performed in a final volume of 50 µl containing DNA (100 ng), 250 µM of each dNTP, 2.5 mM of $MgCl_2$, 20 pmol of each primer, 5 µl of 10X PCR buffer and 1.25U of TaqDNA polymerase (MBI, Fermentas, Lithuania). The PCR products were separated on agarose gels (1.4%) and stained with ethidium bromide. Individual amplicons represented single bands on agarose gels, indicating the specificity of the PCR and the conditions used.

Fragments of the mt-CO1 gene were succesfully amplified using the JB3/JB4.5 primers. Bands were excised from the gel and amplified DNAs were purified with the use of QIAquick Gel Extraction Kit (Qiagen). The mt-CO1 sequences were automatically obtained by ABI PRISM Sequence Detection System and nucleotide sequence analysis was undertaken by BLAST algorithms.

Analysis and phylogenetic tree construction of the obtained sequences of the mt-CO1 gene were performed with CLC Main Workbench software (Knudsen *et al*., 2007). The phylogenetic tree was built using the neighbour-joining method (Saitou and Nei, 1987). The reliability of the inferred tree was evaluated by bootstrap analysis on 1000 replicates. GenBank accession numbers of the reference strains of *E. granulosus* for mt-CO1 used in the phylogenetic analysis are as follows: JF828337 (G1), M84662 (G2), DQ856466 (G3), M84664 (G4), M84665 (G5), M84666 (G6), M84667 (G7), AB235848 (G8) and AF525457 (G10). *Taenia asiatica* (AB465211) was used as an out group.



Figs 1A–1D. A – The cysts (arrows) bulge from paranchyma in visceral surface of liver, Bar:4 cm; **B –** Liver hydatid cysts' diameters ranging from 1 to 5 cm; **C –** Protoscoleces in the cyst fluid; **D –** The cyst wall composed of a peripheral collagenous tissue (arrows) lined by a prominent, slightly lamellar layer of lightly basophilic, acellular material (arrow heads) in spleen

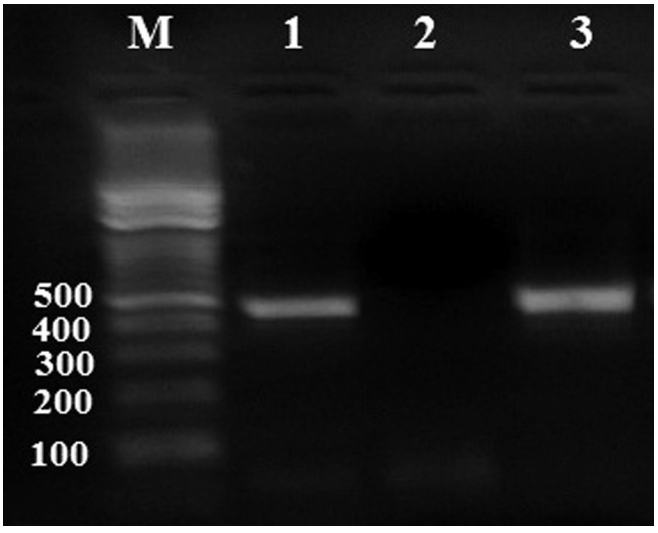


Fig. 2. PCR products of mt-CO1 gene. M: marker (100 bp); 1: positive control; 2: negative control; 3: mule isolate.

On gross examination; the liver was atrophic in appearance and there were 5 and one unilocular cysts, filled with clear fluid in liver and spleen, respectively. Hepatic cysts were bulged from visceral surface of the liver (Fig. 1A and B) and the spleenic cyst was detectable in both surfaces. They were measured as 2x3x3.5, 1x2. Cysts were one-sectional and surrounded by a thick granulation tissue. Spleen cyst could be seen both from parietal and visceral faces of the organ. Cysts fluid was aspirated and examined microscopically for the presence of protoscoleces and/or hooks. All cysts were found to be fertile (Fig. 1C). Histopathologically, the cysts were composed of a peripheral rim of collagenous tissue (host tissue) lined by a prominent, slightly lamelar layer of lightly basophilic, acellular material. Peripheral collagenous tissue was focallly calcified (Fig. 1D).

The 12S rRNA-PCR with the *E.g.*ss1fw and *E.g.*ss1rev primers did not yield a 254 bp of amplification product with the sample analyzed except positive control. However mt-CO1-PCR yielded a 446 bp sized amplification product. PCR amplified mt-CO1 fragments from all isolates are shown in Fig. 2. Sequence corresponding to mt-CO1 gene was identical to a sequence reported for *E. equinus* (formerly G4) (Genbank accession number: KC953029) (Fig. 3).

To date, only two species of Echinococcus have been reported in both human and livestock in Turkey, *E. granulosus sensu stricto* (G1-G3 complex) and *E. canadensis* (G6 and G7 genotypes) (Utuk *et al*., 2008; Snabel *et al*., 2009; Simsek *et al*., 2010; Simsek *et al*., 2011b). Molecular characterization of *E.granulosus* in a horse isolate in Turkey, was firstly reported by Utuk and Simsek, 2013.

It was reported that the equids are intermediate hosts for *E. equinus* (Jenkins *et al*., 2005). The present study identified the genotype of the hydatid cyst isolate obtained from a mule by sequencing a fragment of mt-CO1 gene. In the present study, the molecular characterization of the genotype by sequencing a fragment of mt-CO1 gene revealed the presence of the G4 strain in the mule. This result is in accordance with the report of Bowles *et al*. (1992) who found that *E. granulosus* G4 genotype was the causative agent of cystic echinococcosis in equids by sequencing fragments of CO1 (366 bp)

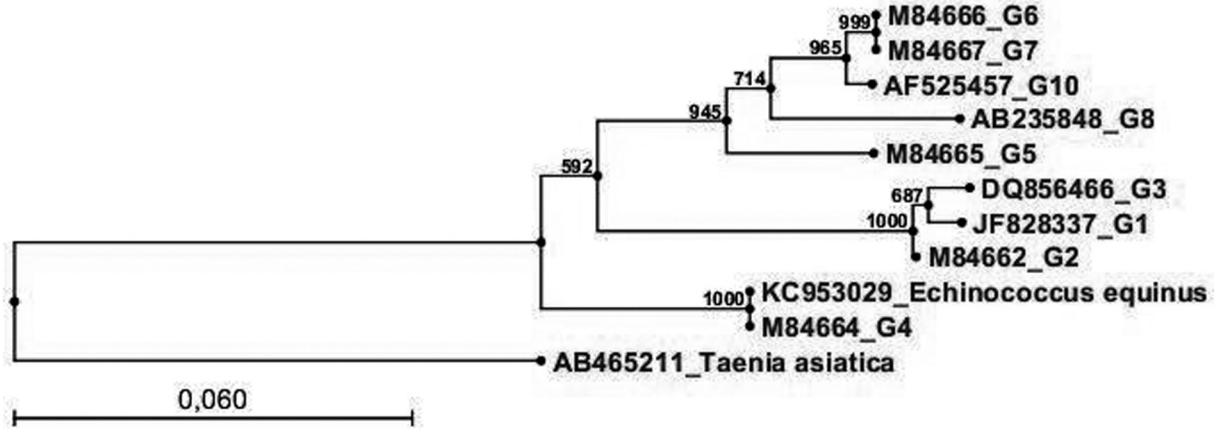


Fig. 3. Phylogenetic tree construction of the obtained sequence of the mt-CO1 gene

and ND1 (471 bp) genes. Recently, a hydatid cyst isolated from the lungs of a mare was identified as *E. equinus* in southern Germany (Blutke *et al*., 2010). Varcasia *et al*. (2008) identified both *E. equinus* and *E. granulosus s.s.* in horses, although cysts of the latter showed aberrant morphological characters and were never fertile. Utuk and Simsek (2013) also reported the *E. granulosus s.s.* in a horse hydatid cyst isolate in Turkey.

It was stated that *E. equinus*, although having a lower occurrence, shows a very high rate of fertility and very well-developed hydatid cysts (Varcasia *et al*., 2008). Ponce *et al*., (1998) reported that the parasite is usually unable to develop into fertile cysts and only small caseous cysts can be observed in infected animals. In the present study we detected a great number of protoscoleces in the cyst fluid.

Certainly, the active pathway of infection in mules remains unclear, species of the infected definite host responsible for fecal contamination of pasture with *E. equinus* eggs is not known. In this respect, contact with feces of an *E. equinus* infected stray dog and more likely wild carnivores (e.g., foxes), which might have had access to infectious equine offal, have to be taken into consideration. Thus, more data on the prevalence of equine hydatidosis in Turkey is needed to better understand the routes of transmission. Moreover, more data on the prevalence of adult worm burdens in the definitive hosts is needed, with special regard to wild carnivores. Therefore, every suspect case of equine hydatidosis should be necropsied. Following to the morphological diagnosis of metacestode stages in equids, identification of definitive strain, species, or both by genetic sequence analysis should be performed. By this way, the dynamics of transmission and the other possible infection routes could be explained.

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