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Detection of *Echinococcus granulosus* G3 in a Wild Boar (*Sus scrofa*) in Central Italy using PCR and Sequencing

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ABSTRACT: We report cystic echinococcosis in a free-living wild boar (*Sus scrofa*) in Europe. Parasites were identified by histopathology and molecular techniques, revealing *Echinococcus granulosus* of the G3 genotype.

Cystic echinococcosis (CE) is a parasitic zoonosis that occurs worldwide, particularly in the Mediterranean basin (Torgerson and Budke 2003; Rojo-Vazquez et al. 2011). The causative agent is *Echinococcus granulosus*, a cestode of the family *Taeniidae*. This parasite occurs mainly in domestic animals with dogs (*Canis lupus familiaris*) as the definitive host. However, *E. granulosus* has many intermediate accidental hosts, including humans.

In Italy, CE is becoming a significant public health problem, particularly in the islands and in the southern regions of the country. Brundu et al. (2014) demonstrated an increased risk of human CE in rural areas with traditional and ancient farming methods, where small ruminants are bred through extensive systems and sheepdogs have access to carcasses or offal of dead livestock often containing hydatid cysts with viable protoscoleces. *Echinococcus granulosus* mainly has a domestic dog–sheep (*Ovis aries*) cycle, but has also a sylvatic wolf (*Canis lupus*)–wild ruminant cycle. These cycles are linked when dogs consume the carcasses of hunted game and when wolves consume carrion or the prey of grazing animals (Gori et al. 2015). According to recent taxonomic studies, *E. granulosus* has been classified into different species by genotyping: *E. granulosus* sensu stricto (sheep G1, Tasmanian sheep G2, water buffalo [*Bubalus bubalis*] G3), *Echinococcus equinus* G4, *Echinococcus ortleppi* G5, *Echinococcus canadensis* G6–10, and *Echinococcus felidis* (Thompson 2008; Casulli et al. 2012; McManus 2013). However, little is known about the factors determining host specificity or developmental differences among the strains (Thompson 2008).

Few epidemiologic studies have evaluated CE in Italian domestic animals. The G1, G2, G3, G4, and G7 genotypes have been detected in southern and island livestock (Busi et al. 2007; Varcasia et al. 2008; Grosso et al. 2012). Given the wide distribution of strains in wildlife populations, particularly in wild boar (*Sus scrofa*; Daniel Mwambete et al. 2004; Gortazar et al. 2007), along with their role in the diffusion of CE (Onac et al. 2013; Sarkari et al. 2015) and their high prevalence in Italy, we report the occurrence of the G3 strain in a wild boar.

During the winter hunting season of 2013–14, a male adult wild boar was culled in the province of Ascoli Piceno (Marche region, Italy). The lungs and intrapulmonary lymph nodes were submitted by the National Veterinary Service at the municipal slaughterhouse for official inspection. The organs were delivered to the Istituto Zooprofilattico Sperimentale, Umbria Marche, Italy for further investigation.

Macroscopic and histopathologic examinations were performed. Portions of the lung and the tracheobronchial and mediastinal lymph nodes were fixed in 10% neutral-buffered formalin. Samples were embedded in paraffin and stained with H&E.

Molecular analysis was performed at the Italian National Reference Center for Echinococcosis. Parenchyma, cysts, and fluid were submitted for DNA extraction for

molecular analysis. Total genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was eluted and stored at –20 C until PCR amplification. A region within the cytochrome c oxidase subunit 1 (*cox*1) gene was amplified using primers CO1.F (5′-TTT.TTT.GGC.CAT.CCT.GAG.GTT.TAT-3′) and CO1.R (5′-TAA.CGA.CAT.AAC.A-TA.ATG.AAA.ATG-3′) (Casulli et al. 2012).

The reactions were performed in automated DNA thermal cyclers (GeneAmp PCR Systems 2400 and 9700; Applied Biosystems, Foster City, California, USA). The PCR products, 460-base pair segments, were verified by electrophoresis on a 1.5% agarose gel stained with SYBR® safe DNA gel stain (Invitrogen, Eugene, Oregon, USA) and examined under ultraviolet transillumination. The PCR products were purified using a QIAquick Spin PCR purification kit (Qiagen) and sequenced using a DNA sequencing kit (dRhodamine Terminator Cycle Sequencing Ready Reaction; Applied Biosystems) according to the manufacturer's instructions. The sequences were edited and aligned using ChromasPro 1.7.7 and compared to "[G1]_AB033407_Reference sequence" in the GenBank database.

Macroscopic examination of the lungs revealed four cysts that were spherical, turgid, fluid-filled, and 20–50 mm in diameter. Histopathologic analysis revealed granulomatous lesions represented by unilocular cystic structures surrounded by an extensive inflammatory reaction induced by eosinophils, lymphocytes, macrophages, and multinucleated giant cells. The outer cyst wall was composed of a hyalinous thick laminated layer that was irregularly folded and contiguous to a thin germinal layer; there were no protoscolices, and only germinal layer cells were found.

To determine the strain of *E. granulosus*, the isolate sequence was aligned and compared to mitochondrial *cox*1 gene sequences of *E. granulosus* available in GenBank database. A basic local alignment search tool (NCBI 2016) analysis showed that the DNA sequence belonged to the *E. granulosus* G3 genotype. Several epidemiologic studies of CE in wild boar have been conducted in Europe; mostly *E. granulosus* sensu stricto G1 and *E. canadensis* G7 have been found (Moks et al. 2008; Rojo-Vazquez et al. 2011; Onac et al. 2013). To the best of our knowledge, this is the first report of the G3 strain in a wild boar in Europe. Although based on one capture, it demonstrates that deeper epidemiologic studies are needed to confirm the ability of *E. granulosus* to cross from the sylvatic to the domestic cycle and vice versa.

In Italy, *E. granulosus* G1–G3 strains were recently isolated from wild wolves (*Canis lupus italicus*) from the Apennine region (Gori et al. 2015). Those authors stressed the importance of wild species as possible indicators for CE environmental spreading. They concluded that the typical behavior of wolves, characterized by long-distance dispersal and wide home ranges and often with overlapping ranges of sheep and wild boar, could promote parasite spread from areas with high ovine CE prevalence. Sheep bred in nonconfined systems, as well as economic, social, and cultural aspects, can lead to "cross-talk" between wild and domestic cycles. In this scenario, the genotyping of *E. granulosus* isolates from humans and domestic and wild animals is essential for assessing the epidemiologic situation of CE in Europe and for better defining the role of wild hosts in the transmission of CE.

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