Veterinary Parasitology 226 (2016) 138–144

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

# Molecular identification of *Echinococcus granulosus* isolates from ruminants in Greece

Erifylli Roinioti[a,b,*], Aegli Papathanassopoulou[c], Ioanna Theodoropoulou[a], Sami Simsek[d], Georgios Theodoropoulos[a]

[a] *Department of Anatomy and Physiology of Farm Animals, Faculty of Animal Science and Aquaculture, Agricultural University of Athens, 75 Iera Odos, Votanikos, Athens 11855, Greece*
[b] *Ministry of Rural Development and Food of the Hellenic Republic, Organization of Agricultural Vocational Education, Training and Employment O.G.E.E.K.A DIMITRA, 68 Nirvana Odos & Kourtidou, Athens 11145, Greece*
[c] *Department of Biotechnology, Agricultural University of Athens, 75 Iera Odos, Votanikos, Athens 11855, Greece*
[d] *Department of Parasitology, Faculty of Veterinary Medicine, University of Firat, 23119 Elazig, Turkey*

ARTICLE INFO

*Article history:*
Received 21 March 2016
Received in revised form 30 June 2016
Accepted 30 June 2016

*Keywords:*
*Echinococcus granulosus*
Genotype
Haplotype
Ruminants
Greece

ABSTRACT

Cystic echinococcosis is a parasitic disease caused by *Echinococcus granulosus*, a cestode with worldwide distribution. Data on the circulating *Echinococcus granulosus* genotypes in Greek livestock is scant. The aim of the present study was to conduct a genetic analysis of 82 *Echinococcus granulosus* isolates from ruminants in Greece, including areas which until today have not been the subject of studies. The analysis relied on a PCR assay targeting cytochrome c oxidase, subunit 1 gene (CO1), followed by bidirectional sequence analysis of the amplification product. Eighty (n = 80) of the 82 (97.6%) isolates were allocated to *Echinococcus granulosus* sensu stricto (G1-G3) and were classified in 13 distinct haplotypes (9 common and 4 novel) with 12 polymorphic sites. The presence of the dominant haplotype EG1 as was documented in the European populations, was indicated in the country. Almost all regions shared the same common haplotype. In comparison to this predominant haplotype, the number of the nucleotide changes in all the other haplotypes ranged from 1 to 5. All nucleotide changes proved to be transitions (A↔G or C↔T). Two fertile hydatid cysts of sheep origin in different areas (Arkadia, Ilia) of the Peloponnese were identified as *Echinococcus canadensis* (G7 genotype).



## 1. Introduction

Cystic echinococcosis (CE) is a parasitic disease caused by the platyhelminth *Echinococcus granulosus,* a cestode with high host adaptability and worldwide distribution (Schantz et al., 1995). Control programmes have been implemented for many years, nevertheless, eradication of the disease, especially in the less developed parts of the world, is still considered a great challenge (Larrieu and Zanini, 2012).

The classification of *Echinococcus* spp. has been a contentious issue for several decades, however, the parasite taxonomy and the species composition within the genus have now almost been clarified (Nakao et al., 2013). According to the currently approved classification, *Echinococcus granulosus* (sensu lato) is subdivided into *E. granulosus* sensu stricto that includes the formerly identified genotypic variants G1-3, *Echinococcus felidis* (the former "lion strain"), *Echinococcus equinus* (the "horse strain", genotype G4), *Echinococcus ortleppi* (the "cattle strain", genotype G5) and *Echinococcus canadensis.* The latter species shows the highest diversity and is composed of the "camel strain", genotype G6, the "pig strain", genotype G7 and two "cervid strains", genotypes G8 and G10 (Romig et al., 2015).

In Greece, a surveillance system based on the inspection of all carcasses at slaughterhouse level and the preventive treatment of dogs with antiparasitic tablets, is in place (Hellenic Republic Ministry of Rural Development and Food, http://www.minagric.gr; European Food Safety Authority, efsa, 2013). According to the data of the last decade, the prevalence of CE in small ruminants in the country was estimated to range between 14% in sheep and goats younger than 2 years and 47% in sheep and 20% in goats, older than 8 years, with a total prevalence of 30.4% and 14.7% in sheep and goat population examined, respectively (Varcasia et al., 2007). Christodoulopoulos et al. (2008) reported a prevalence of 4.43% in hoggets (sheep up to the age of 1 year) and 55.6% in adult sheep

* Corresponding author. Tel.: +30 210 5294381.
*E-mail address:* er@aua.gr (E. Roinioti).

http://dx.doi.org/10.1016/j.vetpar.2016.06.040
0304-4017/

with a total prevalence of 39.32% in sheep population examined. According to the latest data, the prevalence was recorded 30.2% for sheep, 7.86% for goats, 42% for water buffaloes and, 1.1% and 0% for the freely reared wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*), respectively (Chaligiannis et al., 2015), although information on the age of the examined animals is lacking.

The wild faunal richness of Greece is mainly restricted to the wolf (*Canis lupus*), the fox (*Vulpes vulpes*) and the Golden jackal (*Canis aureus* L.), with the jackal being the most rare of the wild canid species in the country (Giannatos et al., 2005). Even though these species are potential hosts, however, we remain largely ignorant of their contribution to the epidemiology of the parasite in the country. In a survey conducted in 1989, which included 580 foxes, 28 wolves, 11 jackals and 4 wild cats captured from the wild, only the wolves were found to be naturally infected at a rate of 14.3% (4/28 positive samples) (Papadopoulos, 1989). Finally, little is known about the infection of dogs in the country. Between the years 1985 and 1987 the National Veterinary Services conducted a mass assessment of 110,093 dogs by the arecoline technique and 3.3% of these animals were found to be infected with *E. granulosus* (Papadopoulos, 1989).

The inclusion of cystic echinococcosis in the list of notifiable diseases varies across countries (Budke et al., 2013). In Greece, according to the National Action Plan for the prevention of communicable diseases 2008–2012 (Hellenic Republic Ministry of Health, www.moh.gov.gr), there is a mandatory notification system of the disease within week (reporting time period following diagnosis). For the period 2005–2009, a total of 79 echinococcal human cases were officially reported with a mean annual notification rate 0.14/100,000 population (Hellenic Centre for Disease Control and Prevention, HCDCP, www.keelpno.gr). According to the latest data recorded in the country, the confirmed echinococcal human cases for the period 2010–2013 were 11 (2010), 17 (2011), 21 (2012) and 10 (2013) (Hellenic Centre for Disease Control and Prevention, HCDCP). However, the real number of cases might be higher due to underreporting (Mead, 1999; Gkogka et al., 2011) and the long asymptomatic period of the disease, a fact that could also partially explain the higher prevalence in elderly (Hellenic Centre for Disease Control and Prevention, www.moh.gov.gr; European Centre for Disease Prevention and Control ECDC, 2014, www.ecdc.europa.eu).

The broad host range and the wide geographic distribution of *E. granulosus* renders typing of isolates significant, in terms of effective disease monitoring and control (Macpherson, 1983; Bourée, 2001; Rausch, 2003; Armua-Fernandez et al., 2014; Poglayen et al., 2014; Scioscia et al., 2016). Genetic typing of *E. granulosus* from livestock in the country was performed in the past on hydatid cysts isolated in slaughterhouses of the Peloponnese and central and northern Greece (Varcasia et al., 2007; Chaligiannis et al., 2015). Regarding those results, among the *Echinococcus granulosus* sensu lato species, *E. granulosus* sensu stricto and *E. canadensis* G7 genotype were found to be present in the country. The aim of the present study was to conduct a genetic analysis of *E. granulosus* isolates from ruminants in the country, including areas which until today have not been the subject of studies and establish an accurate description of their genetic diversity. The results were compared with those reported in the two previous studies and those reported worldwide.

## 2. Materials and methods

### 2.1. Sample collection

The study defines an isolate as a single hydatid cyst from each separate carcass. A total of 82 isolates from adult animals (sheep n = 75, goats n = 6, cattle n = 1) aged between 3 and 7 years and originating from 10 prefectures of mainland Greece and the islands of Euboea and Naxos were collected over a period of 3 years (April 2011- July 2014) during randomly repeated visits to licensed slaughterhouses in Greece. The isolates were preserved in ethanol (70% v:v) and were transferred to the laboratory for fertility assessment and DNA isolation.

### 2.2. Fertility assessment

Fertility assessment was performed by microscopic examination of wet unstained preparations of the hydatid fluid, or when the volume of the latter was not sufficient, of the germinal layer of the hydatid cysts. Cysts containing protoscoleces and/or hooks were considered fertile (Galindo et al., 2002).

### 2.3. DNA isolation

Total genomic DNA was isolated exclusively from the germinal layers of the hydatid cysts using the NucleoSpin® Tissue Kit (Macherey-Nagel) according to the manufacturer's instructions. Prior to DNA isolation, a part of the germinal layer (100–200 mg) was dissected in small sections and ethanol residue was removed, by repeated (n = 5) wash in 600 μl phosphate buffered saline (PBS, MP Biomedicals). DNA quality was evaluated with regard to purity and integrity by submerged gel electrophoresis followed by image analysis using a Bio-Rad ChemiDoc XRS+ Molecular Imager (Bio-Rad Laboratories Inc.), and by optical density ration (260/280 nm), using a NanoDrop 8000 Spectrophotometer (Thermo Fisher SCIENTIFIC). DNA isolated from the samples was processed for genotyping of *E. granulosus* by PCR and sequence analysis.

### 2.4. Genotyping of Echinococcus granulosus

A first screening of the samples was performed targeting the 12S-rRNA gene according to the methodology described by Dinkel et al. (2004). Genotyping and evaluation of the genetic diversity of the *E. granulosus* isolates relied on the PCR amplification of a 444 bp fragment of the cytochrome c oxidase, subunit 1 gene (CO1) with the use of the primer set JB3 and JB4.5 (Bowles et al., 1992) followed by bidirectional sequence analysis of the amplification product. In short, the reaction was prepared with High Fidelity DNA Polymerase (KapaBiosystems) and contained 10 μl reaction buffer (2 mM $MgCl_2$), 200 μM of each dNTP, 20 pmol of each primer, 5 μl DNA target and PCR grade water (Sigma-Aldrich) to a final volume of 50 μl. Amplification was performed for 38 cycles of 1 min at 94 °C, 1 min at 49 °C, 1 min at 72 °C followed by a final extension step of 10 min at 72 °C.

In all cases PCR was performed with an ABI thermal cycler (Applied Biosystems, ThermoFisher SCIENTIFIC Inc.) and the products were analysed by submerged gel electrophoresis using Bio-Rad ChemiDoc XRS+ Molecular Imager (Bio-Rad Laboratories Inc.). CO1 amplicon bands were excised from the gels and subjected to purification steps using the GeneJET Cel Extraction kit (ThermoFisher SCIENTIFIC). Bi-directional sequence analysis was performed using the respective pair of primers (JB3 and JB4.5), with an ABI 3730XL Genetic Analyser (Cemia S.A., Department of Immunology & Histocompatibility, Faculty of Medicine, University of Thessaly, Greece).

### 2.5. Nucleotide sequence data analysis

An NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) BLAST (Altschul et al., 1990) search primarily confirmed the identity of the sequences with deposited ones of *E. granulosus*. Chromatograms' quality of both strands was evaluated, the edges were trimmed and the ambiguities were corrected in FinchTV 1.4.0 (Geospiza Inc., Seattle WA, USA). Consensus sequences of 396 nucleotides were created using GeneStudio™