ORIGINAL ARTICLE PARASITOLOGY

Molecular characterization of *Echinococcus granulosus* in south-eastern Romania: evidence of G1–G3 and G6–G10 complexes in humans

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Abstract

Echinococcus granulosus is the aetiological agent of cystic echinococcosis (CE), which is a public health problem in many eastern European countries, particularly in Romania, where the infection causes a high number of human and animal cases. To shed light on the transmission patterns of the parasite, we performed a genotyping analysis on 60 cyst samples obtained from patients who live in south-eastern Romania and who underwent surgery for liver or lung CE. DNA was extracted from the endocysts or the cyst fluids, and fragments of cytochrome *c* oxidase subunit I and NADH dehydrogenase subunit I mitochondrial genes (*cox1* and *nd1*, respectively) were amplified by PCR and sequenced. We found that most of the samples analysed (59/60) belonged to the G1–G3 complex (*E. granulosus* sensu stricto), which contains the most widespread and infective strains of the parasite. We also identified the first human patient infected by a non-G1–G3 genotype of *E. granulosus* in this country. As the DNA sequence of this cyst sample showed maximum homology with the G6–G10 complex (*Echinococcus canadensis*), this is, in all likelihood, a G7 genotype, which is often found in pigs and dogs in most countries of eastern and south-eastern Europe.

Keywords: Cystic echinococcosis, *Echinococcus canadensis*, *Echinococcus granulosus*, G1–G3 complex, G6–G10 complex, genotype, strain
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Introduction

The cestode *Echinococcus granulosus*, a tapeworm of the family Taenidae, is the aetiological agent of cystic echinococcosis (CE), a neglected chronic infection with a worldwide distribution [1]. In the life cycle of the parasite, the dog and other canids are the definitive hosts, and livestock (especially sheep) are intermediate hosts [2]. Humans are accidental intermediate hosts through the ingestion of parasite eggs, whose larvae can reach almost any organ (mainly the liver and the lung), where they develop and form cysts (metacestodes) [3]. The infection may be asymptomatic for years, and clinical symptoms occur when the cyst exerts pressure on the surrounding tissues/organs, or after their rupture. CE is usually diagnosed with imaging techniques, mainly ultrasound, and, to a lesser extent, by serology [4,5].

Genotyping of human CE is useful to confirm the diagnosis and to collect data on parasite transmission patterns and the susceptibility of humans to a particular genotype of *E. granulosus*. Although the taxonomy of the genus *Echinococcus* continues to be a subject of debate, ten *E. granulosus* strains or genotypes (G1–G10) have been described to date [6–8]. Recently, some authors have suggested that these

genotypes should be clustered into four different species: *E. granulosus* sensu stricto (G1, G2 and G3, or G1–G3 complex), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5), and *Echinococcus canadensis* (G6 to G10, or G6–G10 complex) [9–14]. *E. granulosus* sensu stricto includes the genotypes G1 (sheep strain), G2 (Tasmanian sheep strain), and G3 (buffalo strain), and has been found to more frequently infect humans than other species or strains. The horse strain G4 may be non-infective for humans, and very few cases of infections by the cattle strain G5 have been reported [11,15,16]. The camel strain G6, the pig strains G7 and G9 and the cervid strains G8 and G10 are genetically related, and show moderate infectivity for humans [9–12,17,18].

CE is an important public health problem in eastern Europe, in particular in Romania, where CE is highly endemic and is considered to be the most important zoonotic disease [19]. Bart *et al.* [20] demonstrated the sympatry of G1, G2 and G7 genotypes in Romanian livestock (sheep, cattle, and pigs), and detected the common sheep G1 strain in two human cysts. In this work, we aimed: (i) to identify the *E. granulosus* genotypes that cause CE in a cohort of 60 patients from Romania; and (ii) to determine the possible correlations between the patients' epidemiological and clinical data and the genotype of their parasite.

Materials and Methods

Patients and cyst collection

Cyst samples (endocyst or cyst fluid) were obtained from 60 Romanian patients suffering from CE of the liver or the lung. These patients received surgery as treatment for their disease at the Colentina Clinical Hospital, Floreasca Emergency Clinical Hospital and Marius Nasta Institute for Pneumology in Bucharest, Romania, from 2008 to 2011. The study protocol was approved by the local ethical review board, and each subject gave informed consent.

The following epidemiological and clinical data were collected for all patients: age, sex, housing location (whether urban or rural), county of origin, close contact with stray dogs, if any, type of infection (primary or secondary echinococcosis), cyst location, cyst dimension (small, medium or large if the mean diameter was <5 cm, 5–10 cm or >10 cm, respectively), type of surgery (conservative or radical), and treatment with albendazole, if any, before surgery. Liver cysts were classified according to the WHO Informal Working Group on Echinococcosis (WHO-IWGE) standardized sonographic classification, as active (CE1–2), transitional (CE3a–3b) or inactive (CE4–5) cysts [5,21].

All patients were tested for anti-*Echinococcus* antibodies with two commercial ELISA kits (RIDASCREEN *Echinococcus* IgG by R-Biopharm, Darmstadt, Germany and Anti-*Echinococcus granulosus* ELISA (IgG) by Euroimmun AG, Lübeck, Germany) by the laboratory of Parasitology, Eco-Para-Diagnostic SRL in Bucharest. Serology was considered to be positive if the ELISA index of positivity was >1.1 with both ELISA kits. In the same laboratory, every sample was examined by light microscopy and eosin staining to assess the cyst viability (the presence of viable protoscoleces in the cyst fluid). Endocyst samples were fixed in 95% ethanol, and cyst fluids were centrifuged at 500 ***g*** for 30 min. Each sample was stored at −20°C until being used for the molecular analysis.

Molecular analysis

Prior to DNA extraction, a small sample (*c.* 8 mm^3, 10–15 mg) was obtained from the collected endocysts. The ethanol was then removed, and samples were washed twice for 15 min in *c.* 200 μL of phosphate-buffered saline (PBS). Cyst fluids were centrifuged at 3500 ***g*** for 3 min, and pellets were washed twice for 15 min in 100 μL of PBS. Finally, all samples were centrifuged at 3500 ***g*** for 5 min, and PBS was removed. Genomic DNA was extracted from each sample with the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

Fragments of cytochrome *c* oxidase subunit I and NADH dehydrogenase subunit I mitochondrial genes (*cox1* and *nd1*, respectively) were amplified by PCR, with the specific primers previously described (*cox1* [6]; *nd1* [22]). Primers for *nd1* amplification were modified with a nucleotide substitution in both forward and reverse sequences (EgNadhF, 5′-AGATTCGTAAGGGGCCTAACA-3′; EgNadhR, 5′-ACCACTAACTAACTAATTCTCTTTC-3′; nucleotide substitutions are underlined), based on the *nd1* sequence present in GenBank (accession number: EF367340).

Amplification was performed in a 20-μL final volume containing template DNA (1–10 ng), 0.2 mM premixed solution of dNTPs, 1 μM each primer, 1X PCR buffer, and 0.5 U of Taq DNA polymerase (GoTaq DNA Polymerase; Promega, Madison, WI, USA). The thermal profile was as follows: 2 min at 95°C, 40 cycles of 45 s at 95°C, 45 s at 57°C and 1 min 30 s at 72°C, followed by 10 min at 72°C. The previous PCR conditions were the same for both genes. After gel electrophoresis, PCR products were purified with the Wizard DNA Clean-Up System (Promega), and sequenced.

Analysis of nucleotide sequence data was performed with BLAST algorithms and databases from the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov).

Results

Patients and cysts

A total of 60 cyst samples (34 endocysts and 26 cyst fluids) were obtained from patients who underwent surgery to remove a cyst located either in the liver (83%) or in the lung (17%). The patients came from 19 different counties of south-eastern Romania (Fig. 1), where they lived in either urban (58%) or rural areas (42%), and most of them (97%) had close contact with stray dogs.

Forty-four and 16 patients suffered from primary and secondary cystic echinococcosis, respectively, with cysts being mainly of medium (47%) or large size (47%). Only 25 patients were treated with albendazole before surgery, and treatment was conservative in most cases (92%). Of the 50 liver cysts, 23 were CE1, 19 were CE2, three were CE3a, and five were CE3b, according to the WHO-IWGE sonographic classification of echinococcal cysts. Classification of the ten lung cysts was not available.

Serological investigation performed by ELISA before surgery gave positive results in 44 patients with liver cysts and in only four patients with lung cysts. Light microscopy examination of the cyst fluids allowed for the assessment of protoscoleces in 55 samples.

Other epidemiological and clinical details of the patients are summarized in Table 1.

Molecular analysis

After DNA extraction, *coxI* and *ndI* were amplified as described in Materials and Methods, and PCR products were purified and sequenced. The obtained *coxI* and *ndI* nucleotide sequences from 59 of 60 patients showed maximum homology (>99%) with the G1 and G3 genotype sequences registered in GenBank, and were classified as belonging to the G1–G3 complex (*E. granulosus* sensu stricto). Only one sequence (accession number: HE819406) showed maximum homology (>99%) with the *E. canadensis* complex (G6–G10). The cyst with this G6–G10 genotype came from a 52-year-old woman who underwent radical surgery to remove a medium-sized CE1 cyst located in the liver; she lives in an urban area, and reported frequent contact with stray dogs; data regarding travel abroad were not available.

The striking prevalence (98%) of the G1–G3 complex among the samples analysed prevented us from evaluating any correlations between the epidemiological and clinical data of the patients and the genotype of their cysts.

Discussion

Romania is an eastern European country where CE is hyperendemic and still causes health and economic problems. Neghina *et al.* [19] reported that CE is considered to be the most important zoonotic disease in Romania, because of the

FIG. 1. Geographical origin of the cystic echinococcosis patients. Map of Romania with the counties (in grey) where patients lived. The number of collected samples for each county is indicated.

TABLE 1. Epidemiological and clinical details of the 60 patients

	No.	%
Age (years)		
Maximum	78	–
Minimum	18	–
Mean	45.5	–
Sex		
Male	26	43
Female	34	57
Housing location		
Urban	35	58
Rural	25	42
Contact with stray dog		
Reported	58	97
Not reported	2	3
Type of infection		
Primary CE	44	73
Secondary CE	16	27
Cyst location		
Liver	50	83
Lung	10	17
Cyst size		
Large (>10 cm)	28	47
Medium (5–10 cm)	28	47
Small (<5 cm)	4	6
Type of surgery		
Conservative	55	92
Radical	5	8
Therapy with albendazole		
Administered	25	42
Not administered	35	58
Serology		
Positive	48	80
Negative	9	15
Doubtful	1	2
Not performed	2	3
Viable protoscoleces		
Present	55	92
Absent	5	8
Sample analysed		
Cyst wall	34	57
Cyst fluid	26	43

CE, cystic echinococcosis.

higher number of human and animal cases than in western European countries. Among the different actions that can be carried out to control the transmission of *E. granulosus* and reduce the likelihood of infection, genotyping of animal and human cases of CE may have an important role. The first genotyping study by Bart *et al.* [20] identified three main genotypes of *E. granulosus* circulating in the livestock of four Romanian counties: the common sheep strain (G1), which has spread among sheep and cattle and has been reported in two human CE cases; the Tasmanian sheep strain (G2), which is infective for sheep and cattle; and the pig strain (G7), mostly found in pigs. To shed light on the possible relevance of these *E. granulosus* genotypes in human cases of CE, we performed a genotyping analysis on 60 Romanian patients who underwent surgery for liver or lung CE. This is the first report of genotyping performed on a large sample of human CE patients, who originated from 19 different counties of south-eastern Romania.

We found that most of the samples analysed belonged to the G1–G3 complex. This is unsurprising, given that the common sheep strain (G1) is the most diffuse worldwide among infected humans, and that the G2 and G3 strains are highly infective for humans as well [10,11]. The large number of *E. granulosus cox1* and *nd1* sequences registered in GenBank and the fact that some of the sequences with the highest similarity with ours were described as the G1 genotype, others as the G3 genotype, and others as the G1–G3 complex, prevented us from classifying our samples as belonging to either the G1, G2 or G3 genotype separately. Therefore, we identified our samples as belonging to the G1–G3 complex, following suggestions by some authors that the G1, G2 and G3 genotypes should be clustered into a single species, *E. granulosus* sensu stricto, as they have a worldwide distribution, do not show intermediate host specificity, and occur sympatrically [9–14].

Interestingly, one of the sequences obtained from the cyst samples belonged to the *E. canadensis* complex (G6–G10); in particular, the highest similarity was found with both the G6 (camel strain) and G7 (pig strain) sequences registered in GenBank. The G6 genotype has been demonstrated to infect humans in South America, Africa, and Asia [23], and two cases of human infection in Turkey were reported by Simsek and Kaplan [17]. The G6 genotype was also found in goats in Kenya and even in Argentina, where camels are not present [24–26]. Thus, goats might be involved in the life cycle of the parasite in Romania. On the other hand, the G7 genotype is often found in pigs and dogs in most countries of eastern and south-eastern Europe, and even though human cases are rare [10,11], 33 cases of human infection by the pig strain in people from Austria, Serbia, Macedonia and Hungary were recently reported [18]. Our sequence represents, in all likelihood, the G7 genotype, given its sympatry with the G1 and G2 genotypes in Romania [20], but, to avoid controversy, we classified the cyst sample as G6–G10 complex following molecular epidemiology studies that clustered those genotypes into a single complex or species, namely *E. canadensis* [9–12].

As the great majority of samples belonged to the G1–G3 complex, no correlation between the patients' epidemiological and clinical data and the genotype of their cysts could be evaluated. Nevertheless, some comments on the transmission pattern of CE can be made. Even though most patients (58%) live in urban areas, the main risk factor for contracting CE infection seems to be close contact with stray dogs, as seen in 97% of patients. In the last decades, the free circulation of stray dogs has been an increasing public health problem in Romania that, among others, can contribute to maintaining the high prevalence of human CE infections both in humans and in animals. As reviewed by Neghina *et al.* [19], the average prevalence of CE infection in dogs was quite high

(21.6%; range, 0–83%) during the observation period 1956–1992. The same authors also reviewed studies carried out between 1983 and 2004, which showed a high prevalence of CE infections in livestock: 12.65–92.9% in sheep, 18.98–43.6% in cattle, and 3.81–73.8% in pigs. Overall, these data reflect active circulation of *E. granulosus* between dogs and other intermediate hosts in Romania, thus increasing the risk of infection for humans.

In conclusion, our results obtained with a substantial sample of patients demonstrate that human CE in Romania is mainly restricted to the *E. granulosus* G1–G3 complex. This study also presents the first human case of infection by a non-G1–G3 genotype of *E. granulosus* in this country. This particular genotype is likely to be the G7 genotype (or *E. canadensis*), consistent with other epizootological findings.

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Transparency Declaration

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References

1. Brunetti E, Garcia HH, Junghanss T. Cystic echinococcosis: chronic, complex, and still neglected. *PLoS Negl Trop Dis* 2011; 5: e1146.
2. Craig PS, McManus DP, Lightowlers MW *et al.* Prevention and control of cystic echinococcosis. *Lancet Infect Dis* 2007; 7: 385–394.
3. Larrieu EJ, Frider B. Human cystic echinococcosis: contributions to the natural history of the disease. *Ann Trop Med Parasitol* 2001; 95: 679–687.
4. Brunetti E, Junghanss T. Update on cystic hydatid disease. *Curr Opin Infect Dis* 2009; 22: 497–502.
5. Brunetti E, Kern P, Vuitton DA, Writing Panel for the WHO-IWGE. Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. *Acta Trop* 2010; 114: 1–16.
6. Bowles J, Blair D, McManus DP. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Mol Biochem Parasitol* 1992; 54: 165–173.
7. Thompson RC, McManus DP. Towards a taxonomic revision of the genus *Echinococcus*. *Trends Parasitol* 2002; 18: 452–457.
8. McManus DP, Thompson RC. Molecular epidemiology of cystic echinococcosis. *Parasitology* 2003; 127 (suppl): S37–S51.
9. Nakao M, McManus DP, Schantz PM, Craig PS, Ito A. A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes. *Parasitology* 2007; 134: 713–722.
10. Thompson RC. The taxonomy, phylogeny and transmission of *Echinococcus*. *Exp Parasitol* 2008; 119: 439–446.
11. Romig T, Dinkel A, Mackenstedt U. The present situation of echinococcosis in Europe. *Parasitol Int* 2006; 55 (suppl): S187–S191.
12. Knapp J, Nakao M, Yanagida T *et al.* Phylogenetic relationships within *Echinococcus* and *Taenia* tapeworms (Cestoda: Taeniidae): an inference from nuclear protein-coding genes. *Mol Phylogenet Evol* 2011; 61: 628–638.
13. Saarma U, Jogisalu I, Moks E *et al.* A novel phylogeny for the genus *Echinococcus*, based on nuclear data, challenges relationships based on mitochondrial evidence. *Parasitology* 2009; 136: 317–328.
14. Nakao M, Yanagida T, Okamoto M *et al.* State-of-the-art *Echinococcus* and *Taenia*: phylogenetic taxonomy of human-pathogenic tapeworms and its application to molecular diagnosis. *Infect Genet Evol* 2010; 10: 444–452.
15. Bowles J, Van Knapen F, McManus D. Cattle strain of *Echinococcus granulosus* and human infection. *Lancet* 1992; 339: 1358.
16. De la Rue ML, Takano K, Brochado JF *et al.* Infection of humans and animals with *Echinococcus granulosus* (G1 and G3 strains) and *E. ortleppi* in Southern Brazil. *Vet Parasitol* 2011; 177: 97–103.
17. Simsek S, Kaplan M, Ozercan IH. A comprehensive molecular survey of *Echinococcus granulosus* in formalin-fixed paraffin-embedded tissues in human isolates in Turkey. *Parasitol Res* 2011; 109: 411–416.
18. Schneider R, Gollackner B, Schindl M, Tucek G, Auer H. *Echinococcus canadensis* G7 (pig strain): an underestimated cause of cystic echinococcosis in Austria. *Am J Trop Med Hyg* 2010; 82: 871–874.
19. Neghina R, Neghina AM, Marincu I, Iacobiciu I. Epidemiology and epizootology of cystic echinococcosis in Romania 1862–2007. *Foodborne Pathog Dis* 2010; 7: 613–618.
20. Bart JM, Morariu S, Knapp J *et al.* Genetic typing of *Echinococcus granulosus* in Romania. *Parasitol Res* 2006; 98: 130–137.
21. WHO-IWGE. International classification of ultrasound images in cystic echinococcosis for application in clinical and field epidemiological settings. *Acta Trop* 2003; 85: 253–261.
22. Bowles J, McManus DP. NADH dehydrogenase 1 gene sequences compared for species and strains of the genus *Echinococcus*. *Int J Parasitol* 1993; 23: 969–972.
23. Casulli A, Zeyhle E, Brunetti E *et al.* Molecular evidence of the camel strain (G6 genotype) of *Echinococcus granulosus* in humans from Turkana, Kenya. *Trans R Soc Trop Med Hyg* 2010; 104: 29–32.
24. Wachira TM, Bowles J, Zeyhle E, McManus DP. Molecular examination of the sympatry and distribution of sheep and camel strains of *Echinococcus granulosus* in Kenya. *Am J Trop Med Hyg* 1993; 48: 473–479.
25. Kamenetzky L, Gutierrez AM, Canova SG *et al.* Several strains of *Echinococcus granulosus* infect livestock and humans in Argentina. *Infect Genet Evol* 2002; 2: 129–136.
26. Soriano SV, Pierangeli NB, Pianciola L *et al.* Molecular characterization of *Echinococcus* isolates indicates goats as reservoir for *Echinococcus canadensis* G6 genotype in Neuquen, Patagonia Argentina. *Parasitol Int* 2010; 59: 626–628.