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ORIGINAL ARTICLE

Echinococcus granulosus sensu lato GENOTYPES IN DOMESTIC LIVESTOCK AND HUMANS IN GOLESTAN PROVINCE, IRAN

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SUMMARY

Cystic echinococcosis (CE) is a globally parasitic zoonosis caused by larval stages of *Echinococcus granulosus*. This study investigated *E. granulosus* genotypes isolated from livestock and humans in the Golestan province, northern Iran, southeast of the Caspian sea, using partial sequencing data of the cytochrome *c* oxidase subunit 1 (*cox1*) and NADH dehydrogenase 1 (*nad1*) mitochondrial genes. Seventy *E. granulosus* isolates were collected from animals in slaughterhouses: 18 isolates from sheep, 40 from cattle, nine from camels, two from buffaloes and one from a goat, along with four human isolates (formalin-fixed, paraffin-embedded tissues) from CE patients of provincial hospitals. All isolates were successfully analysed by PCR amplification and sequencing. The sequence analysis found four *E. granulosus* genotypes among the 74 CE isolates: G1 (78.3%), G2 (2.7%), G3 (15%) and G6 (4%). The G1-G3 complex genotype was found in all of the sheep, goat, cattle and buffalo isolates. Among the nine camel isolates, the frequency of G1-G3 and G6 genotypes were 66.7% and 33.3%, respectively. All four human CE isolates belonged to *E. granulosus sensu stricto*. This study reports the first occurrence of the G2 genotype in cattle from Iran and confirms the previously reported G3 genotype in camels in the same country.

KEYWORDS: *Echinococcus granulosus*; Genotyping; *cox1*; *nad1*; Iran.

INTRODUCTION

The larval stage (metacestode) of *Echinococcus granulosus*, the causative agent of cystic echinococcosis (CE), is the source of a globally distributed zoonotic parasitic disease that causes major medical, veterinary and economic losses in endemic countries, including Iran[1,2].

The adult stage of the cestode resides in the small intestine of carnivores, being the domestic and wild canids, the definitive hosts. The intermediate host that harbours metacestodes (hydatid cysts) in liver, lungs and other organs can be one of the numerous species of domestic and wild ungulates, including sheep, goats, cattle, buffalos, camels. Humans may be infected through the accidental intake of parasite eggs in contaminated water or vegetables, or by direct contact with dogs[3]. CE imposes a considerable economic impact in Iran[4]. A number of studies have estimated the prevalence of CE in Iranian livestock to be between 5.1% and 74.4% in sheep; 3.5% and 38.3% in cattle; 2% and 20% in goats; 11.9% and 70% in buffalos; and between 25.7% and 59.3% in camels[5,6]. Human cases of CE are widespread in Iran and are routinely reported from medical centres and hospitals across the country, including approximately 1% of all surgical cases[6,7].

In the past 50 years, significant phenotypic and genetic variation has been revealed among *E. granulosus* isolates from different intermediate host species in several geographical areas. This led to the establishment of new species and genotypes. The understanding the *E. granulosus* species and genotypes has had a significant impact on the epidemiology and control strategies for the disease, as well as for future vaccine and drug design[8,9]. Based mainly on the *E. granulosus* mitochondrial DNA-based studies, it has been shown that *E. granulosus* comprises 10 genotypes (G1-G10), which have been characterized as distinct species, comprising *E. granulosus sensu stricto* (G1, G2 and G3); *E. equinus* (G4); *E. ortleppi* (G5); and the controversial group formed by G6-G10 species that according to some authors should be regarded as one species, and to others such as the three species namely *E. canadensis*, *E. borealis* and *E. intermedius*. The validity of the G9 genotype is not clear[9-14]. Recently, the lion strain has been proposed as another new species and *E. felidis* was settled as a sister taxon of *E. granulosus sensu stricto* [15].

Several molecular studies performed in Iran have shown the occurrence of *E. granulosus sensu stricto* (G1-G3) and *E. canadensis* (G6)[16-22]. Due to the lack of more accurate data from this region, the present study has investigated the population genetic pattern of

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E. granulosus isolated from livestock and humans by sequencing and the phylogenetic analysis of cytochrome *c* oxidase subunit 1 (*cox1*) and NADH dehydrogenase 1 (*nad1*) mitochondrial genes.

MATERIALS AND METHODS

Collection of samples

The present cross-sectional study was performed from April 2011 to July 2012. Hydatid cysts of liver and lung tissues were collected from sheep, goats, cattle, buffalos and camels, in different slaughterhouses, in Golestan province of northern Iran, southeast of the Caspian sea (Table 1). Cysts that did not contain parasites or calcified cysts were excluded from the study. Molecular techniques were conducted on isolates using clean cyst fluid samples and some whitish germinal layer. Additionally, formalin-fixed paraffin-embedded tissues (FFPT) from patients with histologically confirmed hydatid cysts coming from a private hospital of Gorgan were also evaluated in this study (Table 1). Protoscoleces from individual cysts were aspirated and washed three times with normal saline and preserved at -20 °C until used for the molecular analysis.

DNA Extraction

Isolates underwent four freeze and thaw cycles in liquid nitrogen alternated with a passage in a water bath at 95 °C. Samples were then suspended in 200 µL of tissue lysis buffer and 80 µL of proteinase K and incubated at 56 °C overnight[23]. Subsequently, genomic DNA was isolated from the homogenised suspension using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's recommended protocol.

Extracting DNA from FFPE tissues of human CE was performed using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

A spectrophotometer (NanoDrop® ND-1000, Thermo Scientific, Massachusetts, USA) was used to ensure the quality of DNA extraction. The genomic DNA was kept at -20 °C until amplification.

Individual genomic DNA samples were analysed using amplification of two mitochondrial DNA fragments within *cox1* and *nad1* genes, separately. JB3 (TTTTTTGGGCATCCTGAGGTTTAT) and JB4.5 (TAAAGAAAGAACATAATGAAAAT G) sequences were used as *cox1* forward and reverse primers, and MS1 (AGATTCGTAAGGGGCCTAATA) and MS2 (ACCACTAACTAATTCACTTTC) sequences were used as *nad1* forward and reverse primers, respectively[18]. PCR was carried out in a final volume of 50 µL, including 4 µL (50-100 ng) of genomic DNA, 3.5 mM $MgCl_2$, 250 µM of dNTPs, 25 pmol of each primer and 2 U of Taq polymerase, under the following conditions: 35 cycles of 94 °C for 30 s, 50 °C for 45 s, 72 °C for 35 s, followed by a final extension of 72 °C for 10 min Negative (no added DNA) and positive controls were included in each PCR experiment. PCR products were analysed by electrophoresis in ethidium bromide-stained 1% agarose gels prepared in TBE buffer (65 mM Tris-HCl, 22.5 mM boric acid, 1.25 mM EDTA, pH 9). The gels were visualized using an UV transilluminator (UVitec, Cambridge, UK).

DNA sequencing and phylogenetic analysis

A panel of 74 PCR amplicons for each *cox1* and *nad1* gene was purified and subjected to sequencing in two directions, using the same forward and reverse PCR primers.

The electropherogram of each sequence was visually checked and the sequences were compared to each other and with reference sequences using the BioEdit[24] and the BLAST softwares available at http://www.ncbi.nlm.nih.gov/. The representative sequences for both *cox1* and *nad1* genes were submitted to GenBank.

Three separate phylogenetic analyses of sequencing data were conducted (i) using: pcox1 data for sequences determined in the present study only, and pcox1 data for *T. saginata* as the outgroup; (ii) pnad1 data for sequences determined in the present study only, and pnad1 data for *T. saginata* as the outgroup; (iii) concatenated pcox1+pnad1 data representing all genetic variations detected in the present study, previously described *E. granulosus* genotypes (G1-G10), *Echinococcus* species along with *T. saginata* as the outgroup. The character-based Bayesian inference method (BI) was used for the analyses. BI was executed using the software MrBayes v.3.1.2 available at http://mrbayes.csit.fsu.edu/index.php. Posterior probabilities (pp) were designed for 2,000,000 generations (ngen: 2,000,000; burnin: 20 000) by means of the Monte Carlo Markov Chain method and four simultaneous tree-building chains (nchains:4), with each 100th tree saved (samplefreq:100). The evolutionary distance was determined using the General Time Reversible evolutionary model (nset: 6), allowing for a gamma-shaped variation in mutation rates between codons (rates: g). The TreeviewXv.0.5.0 program[25] was used to indicate the consequence tree.

RESULTS

Seventy-four CE isolate fragments of approximately 450 bp and 400 bp long were successfully amplified within *cox1* and *nad1* genes, respectively. The obtained consensus sequences of *cox1* and *nad1* genes were 366 bp and 378 bp, respectively. Fifty-eight (78.3%), 2 (2.7%), 11 (15%) and 3 (4%) isolates belonged to the G1, G2, G3 and G6

Table 1
Echinococcus granulosus genotypes in different hosts identified by mitochondrial *cox1* and *nad1* sequence analysis in Golestan province, northern Iran

Host (No. of isolates)	Sheep (18)	Goat (1)	Cattle (40)	Buffalo (2)	Camel (9)	Human (4)	Total (74)
Genotypes, No. (%)	G1, 18 (100)	G1, 1 (100)	G1, 29 (72.5) G2, 2 (5) G3, 9 (22.5)	G1, 2 (100)	G1, 4 (44.5) G3 2 (22.5) G6 3 (33.3)	G1, 4 (100)	G1, 58 (78.3) G2, 2 (2.7) G3, 11 (15) G6, 3 (4)

genotypes, respectively. All four human CE isolates belonged to the G1 genotype (Table 1). The sequence alignments of the isolates displayed eight characteristic profiles in *cox1* sequences and 5 characteristic profiles in *nad1* sequences. Sequence profiles for *cox1* (designated as Golc1-8) and *nad1* (designated as Goln1-5) were submitted to GenBank, accession numbers KM513626- KM513633 and KM513634- KM513638, respectively. As some *E. granulosus* sequences were the same in different hosts, the equal sequence profile in different hosts was named as sub-numbers and submitted to GenBank with the related accession numbers KT074941- KT074949 and KT074936- KT074940 for *cox1* and *nad1* genes, respectively. For example Golc6 (AN: KM513631), and Golc6-1 (AN: KT074949), have the same *cox1* sequence in cattle and camel hosts, respectively (Table 2).

Separate phylogenetic analyses of pcox1 and pnad1 data sets were conducted, and all the combinations of *cox1* and *nad1* sequence types, representing all the 74 isolates in the present study were determined. These analyses revealed a concordance between the genotypic classification of pcox1 and pnad1, inferring the utility of combined pcox1 and pnad1 data to access the haplotypic variation among *E. granulosus*. Hence, each pair of pcox1 and pnad1 sequence types (e.g. Golc1- Golc8 and Goln1- Goln5) was used to define the ''working'' haplotypes (see Table 2 and Fig. 1). In all the cases, concatenated reference sequences represented the same isolate (i.e. Golc1 and Goln3 sequences were derived from the same isolate representing the haplotype 2 (H2) in the Table2). A data set representing the concatenated pcox1+pnad1 sequences for all the 15 haplotypes (H1-H15) detected in this study was employed, along with key reference data (comprising concatenated pcox1+pnad1 sequences from previous studies representing all the recognized *Echinococcus* species and *E. granulosus* ''genotypes'', with *T. saginata* as the outgroup; see Table 2 and Fig. 1).

Phylogenetic analyses of concatenated data, for the haplotypes 1-15, were performed by using BI, including representative sequence data for all the recognized species of *Echinococcus* and genotypes of *E. granulosus,* as well as *T. saginata* as an outgroup (Table 2 and Fig. 1). A consensus tree has been built and is shown in Figure 1.

Most of the isolates (78.3%) were identified as G1 and were clustered in the G1 reference genotype (accession nos: *cox1*, U50464; *nad1*, AJ237632). Isolates that were identified as G2 (2.7%) were clustered in the G2 reference sequences (accession nos: *cox1*, M64662; *nad1*. AJ237633). Isolates identified as G3 (14.86%) clustered in the G3 reference sequences (accession nos: *cox1*, M64663; *nad1*. AJ237634) and isolates identified as G6 (13.1%) were clustered in the G6 reference sequences (accession nos: *cox1*, M84666; *nad1*, AJ237637) (Fig.1).

H1-H9 represents the G1 genotype, H10 belongs to the G2 genotype, H11-H14 represents the G3 genotype, and H15 belongs to the G6 genotype. A consensus tree based on the phylogenetic analyses of concatenated *cox1* and *nad1* sequences revealed two distinct clusters. One cluster contained a G1-G3 complex (pp = 1.00) and the other cluster contained the G6-G10 complex (pp = 1.00). Fourteen haplotypes (H1-H14) were found in the G1-G3 cluster, and one haplotype was placed in the G6-G10 complex, the *E. canadensis* (Fig. 1).

DISCUSSION

The results of this study showed that the G1 genotype (*E. granulosus sensu stricto*) was the most prevalent identified genotype among the 74 CE isolates from the Golestan province, northern Iran. The G1 genotype was identified in all of the sheep, goat, buffalo and human isolates. Furthermore, 72.5% of the cattle and approximately half of the camel (44.44%) isolates also belonged to the G1 genotype. Two (5%) and nine (22.5%) of the cattle isolates had the G2 and G3 genotype, respectively. Also, two (22.2%) and three (33.3%) camel isolates showed the G3 and G6 genotypes, respectively.

A recent study performed in this region using the ITS1-RFLP method, has reported the G1 genotype in all the human, sheep and cattle isolates and both, G1 and G6 genotypes, in camel isolates[31]. However, the G1-G3 genotypes cannot be differentiated by the ITS1-RFLP. In another study, all the five cattle and 10 sheep isolates from the Golestan province had the G1 genotype[18]. The finding of the G1 genotype as the most prevalent one suggests that the sheep-dog cycle is dominant in CE from the Golestan province. G1 is the most frequent genotype found in humans and livestock throughout the world[32,33]; however, in some north African countries, including Mauritania and Sudan, G6 is the most common genotype in sheep, cattle, camels and humans[34,35].

Although the *E. granulosus* G2 genotype was primarily introduced as a Tasmanian sheep strain, it was later found in other hosts, including goats, cattle, buffalos, camels and humans, from different countries. In Iran, this genotype has been previously reported and dogs are the definitive hosts. To the best of our knowledge, the present study reports the first occurrence of *E. granulosus* G2 genotype in intermediate hosts in this country.

The *E. granulosus* G3 genotype has been previously reported in humans, sheep, goats, cattle and camels in different countries, including Iran[20,21,36-42]. The present study found this genotype in nine of 40 cattle (22.5%) and two of nine camels (22.2%). However, no sheep, goat, buffalo, or human isolates harboured the G3 genotype in the present study.

Most of the human CE isolates from Iran have been reported as belonging to the G1 genotype. This is consistent with the findings of the present study, as all four human FFPE tissues had the G1 genotype. In a study from the Isfahan province of central Iran, using PCR-RFLP, all 30 human CE isolates were reported as belonging to the G1 genotype[43]. Another study, using SSCP coupled to partial *cox1* and *nad1* sequencing, found that all 23 human CE originally from central Iran had the G1 genotype[18]. A study in the Ardabil province of north western Iran reported seven and two human CE isolates belonging to the G1 and G3 genotypes, respectively[21]. Harandi *et al*., using a PCR-RFLP method, reported 33 and three human CE isolates as belonging to the G1 and G6 genotypes, respectively[16]. The only human CE isolate from the Kerman province of south western Iran showed a G6 genotype[20]. A recent study reported the G6 genotype in all eight FFPE tissues from cerebral *Echinococcus* cysts in patients from a Tehran hospital. The authors also claimed that the G6 genotype has a higher affinity for the human brain than the G1 genotype[44]. In a study on 125 human isolates conducted by Rostami *et al*. (2015), G1 and G6 genotypes were shown to be present in 54.4 and 40.8% of the isolates, respectively. The G6 genotype is especially prevalent in south-eastern parts of the Iran where humans were slightly more infected by camel strains (G6) than G1 strains (45.8 vs. 41.7%). These data show that humans are quite susceptible to infections by the G6 genotype (*E. canadensis*),

Table 2

E. granulosus haplotypes from Golestan Province, Iran and reference of sequences used for concatenation (*cox1*+ *nad1*) and subsequent phylogenetic analyses (see Fig. 1)

***E. granulosus sensu lato* haplotypes from Golestan**	**Host**	**Profile *cox1* (Accession number)**	**Profile *nad1* (Accession number)**	**Reference**
H1	Human sheep cattle	Golc1(KM513626) Golc1-1(KT074941) Goc1-2(KT074942)	Goln1(KM513634) Goln1-1(KT074936) Goln1-3(KT074938)	This study
H2	Human	Golc1(KM513626)	Goln3(KM513636)	This study
H3	Sheep cattle camel	Golc1-1(KT074941) Golc1-2(KT074942) Golc1-3(KT074943)	Goln4(KM513637) Goln4-1(KT074939) Goln4-2(KT074940)	This study
H4	Human sheep cattle	Golc2(KM513627) Golc2-1(KT074944) Golc2-2(KT074945)	Goln1(KM513634) Goln1-1(KT074936) Goln1-3(KT074938)	This study
H5	Cattle	Golc2-2(KT074945)	Goln2(KM513635)	This study
H6	Cattle camel	Golc2-2(KT074945) Golc2-3(KT074946)	Goln4-1(KT074939) Goln4-2(KT074940)	This study
H7	Cattle camel	Golc3(KM513628) Golc3-1(KT074947)	Goln4-1(KT074939) Goln4-2(KT074940)	This study
H8	Goat	Golc4(KM513629)	Goln1-2(KT074937)	This study
H9	Cattle	Golc4-1(KT074948)	Goln4-1(KT074939)	This study
H10	Cattle	Golc5(KM513630)	Goln4-1(KT074939)	This study
H11	Cattle	Golc6(KM513631)	Goln1-3(KT074938)	This study
H12	Cattle camel	Golc6(KM513631) Golc6-1(KT074949)	Goln4-1(KT074939) Goln4-2(KT074940)	This study
H13	Cattle	Golc7(KM513632)	Goln1-3(KT074938)	This study
H14	Cattle	Golc7(KM513632)	Goln4-1(KT074939)	This study
H15	Camel	Golc8(KM513633)	Goln5(KM513638)	This study
E. granulosus sensu lato				
G1	Sheep	M84661	AJ237632	[26,27]
G2	Sheep	M84662	AJ237633	[26,27]
G3	Buffalo	M84663	AJ237634	[26,27]
G4	Horse	M84664	AJ237635	[26,27]
G5	Cattle	M84665	AJ237636	[26,27]
G6	Camel	M84666	AJ237637	[26,27]
G7	Pig	M84667	AJ237638	[26,27]
G8	Moose	AB235848	AB235848	[10]
G10	Reindeer	AF525457	AF525297	[28]
E. felidis	Lion	EF558356	EF558357	[15]
E. multilocularis1	Human	M84668	AJ237639	[26,27]
E. multilocularis2	Rodent	M84669	AJ237640	[26,27]
E. shiquiqus	Pika	AB208064	AB208064	[10]
E. vogeli	Rodent	M84670	AJ237641	[26,27]
E. oligarthra	Rodent	M84671	AJ237642	[26,27]
Outgroup				
Taenia saginata	Cattle	Not available	AJ239106	[29,30]

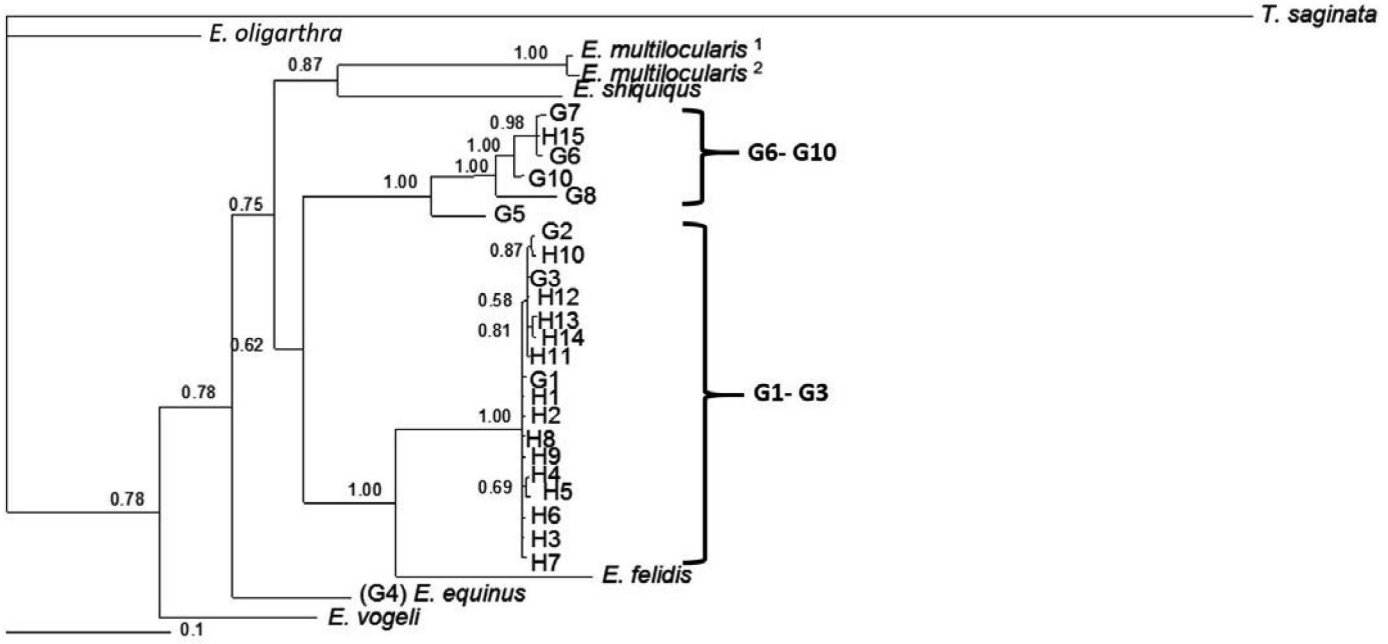

Fig. 1 - Genetic relationships of *Echinococcus granulosus* isolates from the Golestan province; Iranian and reference sequences of *E. granulosus sensu lato* and other species of *Echinococcus* from previous studies, as well as *Taenia saginata* as the outgroup. The relationships were inferred based on phylogenetic analysis of concatenated *cox1* + *nad1* sequence data (H1-H15) using the Bayesian inference (BI). Haplotypes 1-14 represent genotypes G1-G3 (G1-G3 complex, *E. granulosus sensu stricto*), whereas the haplotype 15 represents the genotype G6 (in the G6-G10 complex, *E. canadensis*). The accession numbers and sources of sequences are shown in Table 2. Nodal support is given as a pp value.

and also that there is an active camel-dog cycle in many parts of the country with camel and sheep as potential intermediate hosts. The results of the present study indicate the interaction of the camel-dog and the sheep-dog cycles in this Iranian region[45]. In the present study, two of nine (22.2%) camel isolates had the G6 genotype. This finding is almost entirely in accordance with a previous study conducted on 19 camel isolates from central Iran that found the G6 genotype in 31.6% of isolates, with most of the isolates belonging to *E. granulosus sensu stricto* (68.4%)[17]. Furthermore, the low frequency of the G6 genotype in this study may be the result of low breeding and slaughtering of camels in this region.

In the present study, most isolates designated as haplotypes 1 to14 (H1-H14), formed a strongly supported clade (pp = 1.00) together with reference sequences representing *E. granulosus* G1-G3 genotypes (*E. granulosus sensu stricto*), and the exclusion of *E. felidis* (pp = 1.00). H15 as the only haplotype belonging to the G6 genotype was clustered in the *E. granulosus* G6-G10 genotypes (known as the *E. canadensis* G6 genotype) with a maximal statistical support (pp = 1.00); a strong support was also placed in a smaller cluster in the G6 and G7 genotypes (pp = 0.98).

There are still controversies on the nature of *E. canadensis* (G6, G7, G8 and G10). The G7 (pig strain) is predominantly found in Europe while the G6 genotype has been found in central Asia, the middle east/north Africa and South America with a very distinct epidemiological and biological context in comparison with G8 and G10 genotypes. According to Lymbery *et al*. (2015), G6 and G7 are not believed to be sympatric in most parts of the globe and the nomenclature of G6-G10 genotypes warrants more precise explanation. The division of *Echinococcus granulosus sensu lato* into G-numbers is a relic of the 1990s and should be reconsidered[14,46]. This is especially true for the micro-variants G1-3 and G6/7, whose biological relevance are largely questionable.

The present study found that the predominant genotype is G1 (78.4%), as in other areas of the country, and describes the first report of the G2 genotype identified in cattle hosts. Also, this study confirms previous reports of the G3 genotype in camels and cattle species in the country. As humans can be infected with G2 and G3 genotypes, the epidemiological implication of cattle and camels in maintaining the transmission cycle of different *E. granulosus* genotypes warrants more attention.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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