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Genetic characterization of hydatid cysts of different intermediate hosts

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Summary

Cystic echinococcosis is an important cosmopolitan parasitic zoonosis that causes public health and economic problems in Egypt. The present study was undertaken to identify genotypes of hydatid cyst (HC) DNA isolated from different animal isolates and to identify the genotype of secondary hydatid cysts (HCs) developed in rabbits experimentally infected with camel HC for detection of any genetic mutation. In the present study, we extracted DNA from the germinal layers of 8 HCs collected from 3 camels, 1 cattle, 1 sheep and 3 donkeys in addition to 3 secondary HCs collected from rabbits experimentally infected with camel HC. PCR amplification of the ITS1 gene of all examined samples showed an amplified DNA band at 1115 bp. The partial nucleotide sequences of the ITS1 gene of all isolates were aligned and compared with the reference sequences of the genotypes G1–G8 in GenBank. The camel and rabbit samples were identified as *Echinococcus canadensis* genotype 6 (G6), while the cattle and sheep samples belonged to *E. granulosus sensu* stricto (G1). The donkey isolates belonged to *E. equines* (G4). Alignment of the ITS1 partial nucleotide sequences of the camel HCs and rabbit secondary HCs isolates with the G6 partial nucleotide sequence in GenBank was performed. Both camel HCs and rabbit secondary HCs isolates exhibited the same sequence identity matrix, which indicated the absence of mutation in the rabbit secondary HCs. It can be concluded that camel and rabbit samples were identified as *E. canadensis* (G6), the cattle and sheep samples belonged to *E. granulosus sensu stricto* (G1) and donkey isolates belonged to *E. equines* (G4). No mutation occurred during HCs transmission from camel to rabbit.
Keywords: Hydatid cyst; secondary hydatidosis; PCR; sequencing; mutation

Introduction

Cystic echinococcosis (CE) is one of the most widespread parasitic zoonotic diseases in the world**.** Identification of the cyst genotype would be beneficial for prevention and control of the disease (Ahmed *et al*., 2017). Four species of *Echinococcus* were recognized until a few years ago, namely, *E. granulosus*, *E. multilocularis*, *E. vogeli*, and *E. oligarthus* (Thompson & McManus, 2002). Recently, molecular characterization based on genome pattern, morphology and host specificity differentiated *E. granulosus* species into 10 genotypes (G1-G10) (Madawy *et al*., 2011): *E. granulosus sensu stricto* (*E. granulosus s. s.*) (G1, a common sheep strain; G2, a Tasmanian sheep strain; and G3, a buffalo strain), *E. equinus* (G4, a horse strain), *E. ortleppi* (G5, a cattle strain) and *E. canadensis* (G6, a camel strain; G6/G7, cattle strain; G7, a pig strain; G8, a cervid strain; and G10, a Fennoscandian cer-

* – corresponding author

Table 1. *Echinococcus* species and GenBank accession numbers of the ITS1 gene used for phylogenetic analysis.

HC isolates	*E. granulosus*	Genotype of isolate	GenBank accession no. of isolate	Reference accession no. in GenBank
Camel	*E. canadensis*	G6	MK460268	AJ237775.1
Camel	*E. canadensis*	G6	MK460269	AJ237775.1
Camel	*E. canadensis*	G6	MK460272	KP866147.1
Cattle	*E. granulosus s.s*	G1	MK460273	AY969044.1
Sheep	*E. granulosus s.s*	G1	MK460270	KJ363926.1
Donkey	*E. equinus*	G4	MK460266	AJ237773.1
Rabbit	*E. canadensis*	G6	MK460267	AJ237775.1
Rabbit	*E. canadensis*	G6	MK460271	KP866147.1

vid strain); the G9 strain remains unidentified (Nakao *et al*., 2010, Ohiolei *et al*., 2019). Phylogenetic analysis of nuclear genes such as internal transcribed spacer 1 (ITS1) (Espinoza *et al*., 2014) was used to identify *E. granulosus* genotypes. In North Africa, Eastern Africa and Tunisia, the sheep strain (G1) and camel strain (G6) were found to be the two main genotype groups (Madawy *et al*., 2011). In Sudan, Omer *et al*., 2010 reported that the camel strain was the predominant strain and was infective towards humans. In Egypt, most samples collected from camels slaughtered at the Toukh abattoir (Qalyubia Governorate) were identified as *E. canadensis* (G6), and a few of these strains were identified as *E. ortleppi* (G5), isolated from newly imported Sudanese camels (Abdel Aziz *et al*., 2016). Abbas *et al*., 2016 reported, for the first time, a cattle HC formed by the G1 sheep strain and another cyst formed by the G5 strain (a cattle strain). Additionally, the G1 sheep strain was detected in both Egyptian camel and sheep (Amer *et al*., 2015). Molecular identification of HCs collected from Egyptian donkeys revealed that these cysts were formed by *E. equinus* (G4) (Aboelhadid *et al*., 2013). The present study was undertaken to identify genotypes of HCs DNA isolated from camels, cattle, sheep and donkeys by sequencing of PCR-amplified nuclear ITS1 gene products. Additionally, identification of the genotype of secondary HCs developed in rabbits intra-peritoneally injected with proto-scolices (PSCs) of HCs (of camel origin) to detect the presence of any genetic mutation or variation that occurred during parasite transmission between two intermediate hosts.

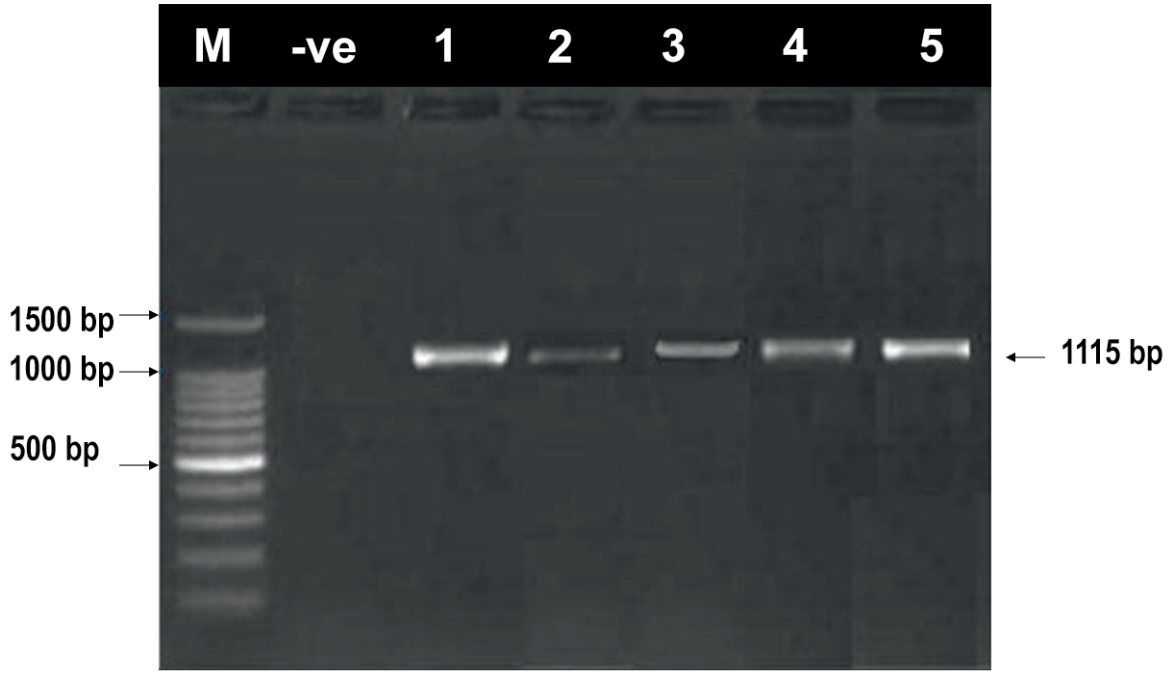

Fig. 1. An ethidium bromide-stained agarose gel (1.5 %) showing the PCR amplification product of the ITSI gene of *E. granulosus*. Lane M: a 100-bp plus molecular size marker; lane –ve: negative control; lane 1: camel host DNA; lane 2: cattle host DNA; lane 3: sheep host DNA; lane 4: donkey host DNA; and lane 5: rabbit host DNA.

Materials and Methods

Hydatid cyst (HC) collection and preparation

Germinal layers from eight HCs (3 fertile cysts from 3 camel lungs, one sterile cyst from cattle liver, one sterile cyst from sheep liver and 3 fertile cysts from 3 donkey livers) were collected and preserved in 70 % ethanol and -20 °C until DNA extraction. In addition, 3 germinal layers were collected from 3 secondary HCs from rabbits, which were also prepared according to Ito *et al*., 2001.

Sixteen male New Zealand white rabbits (1.5 kg in body weight and 4 months old) were divided into 2 groups of eight. Rabbits were reared under good hygienic conditions (clean, well ventilated and warm) and fed a balanced diet. All rabbits were examined to ensure the absence of parasites via coprological examination once daily for 15 days. Each rabbit in the first group received 2 ml of PBS containing 2000 viable PSCs intraperitoneally (Ito *et al*., 2001), while the 2nd group of rabbits was used as a non-infected control negative group. The rabbits were slaughtered on the 13th w.p.i. The different muscles and all visceral organs and surrounding membranes, particularly the lung, liver, kidney, heart and spleen, were examined macroscopically for the presence of HCs. Three germinal layers were collected from 3 secondary HCs from rabbits and prepared as previously described until being used for DNA extraction.

DNA extraction

DNA was extracted using a manual commercial kit (the Thermo Scientific GeneJET Genomic DNA Purification Kit) according to the manufacturer's instructions. DNA was stored at -20 °C until being used for DNA amplification.

PCR amplification

PCR analysis was performed using the BD1 (forward; 5-GTCG-TAACAAG GTTT CCGTA-3) and 4S (reverse; 5-TCTAGATGCGT-TCGAA (G/A) TGTCGATG-3) primers with standard PCR procedures for amplification of the ITS1 gene as described by Jamali *et al*., 2004 and Barghash *et al*., 2017 Amplification was performed in a programmable thermal cycler (Nexus Gradient, Eppendorf, Germany) with an annealing temperature of 55 °C. The corresponding amplicons were checked on a 1.5 % agarose gel. The DNA bands were visualized using ultraviolet transillumination after gel staining with ethidium bromide (0.5 mg/ml).

Sequencing of PCR products

PCR products were purified using the BigDye Xterminator Purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Cycle sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The samples were injected into a 3500 Genetic Analyzer (Thermo Fisher Scientific, USA). DNA sequencing was performed in both directions using the forward and reverse primers for the ITS1 nuclear gene. Nucleotide sequences were first analysed and edited to check electropherogram quality using the software program FinchTV v 1.4.0 (Geospira Inc.©). Nucleotide sequence analysis

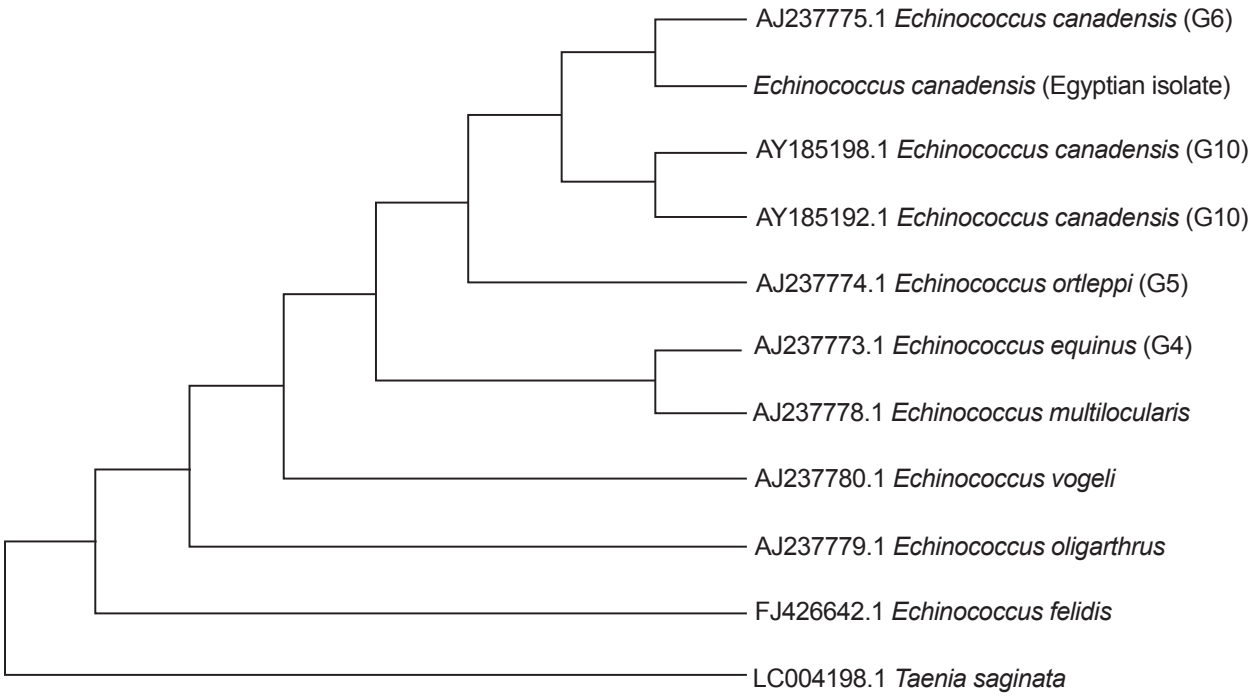

Fig. 2. Genetic relationship between an *E. canadensis* isolate from a camel HC (accession no.: MK460268) and the reference sequences of genotypes G1–G8 based on phylogenetic analysis of ITS1 nucleotide sequences.

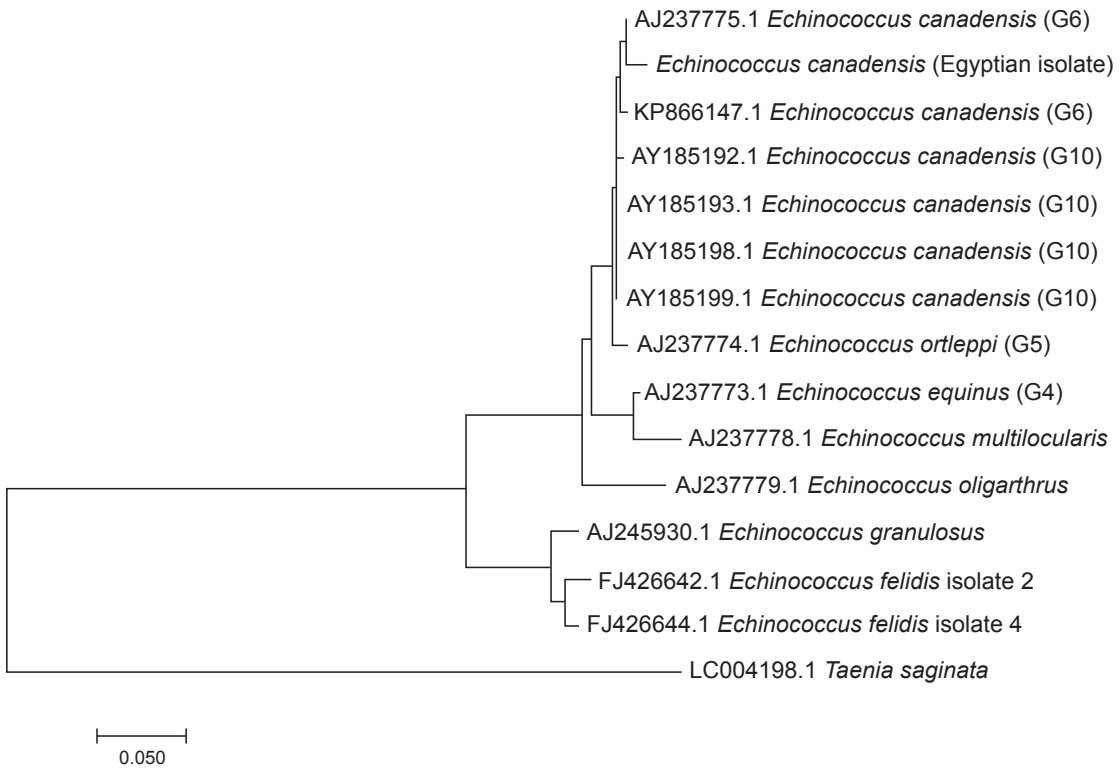

Fig. 3. Genetic relationship between an *E. canadensis* isolate from a camel HC (accession no.: MK460269) and the reference sequences of genotypes G1–G8 based on phylogenetic analysis of ITS1 nucleotide sequences.

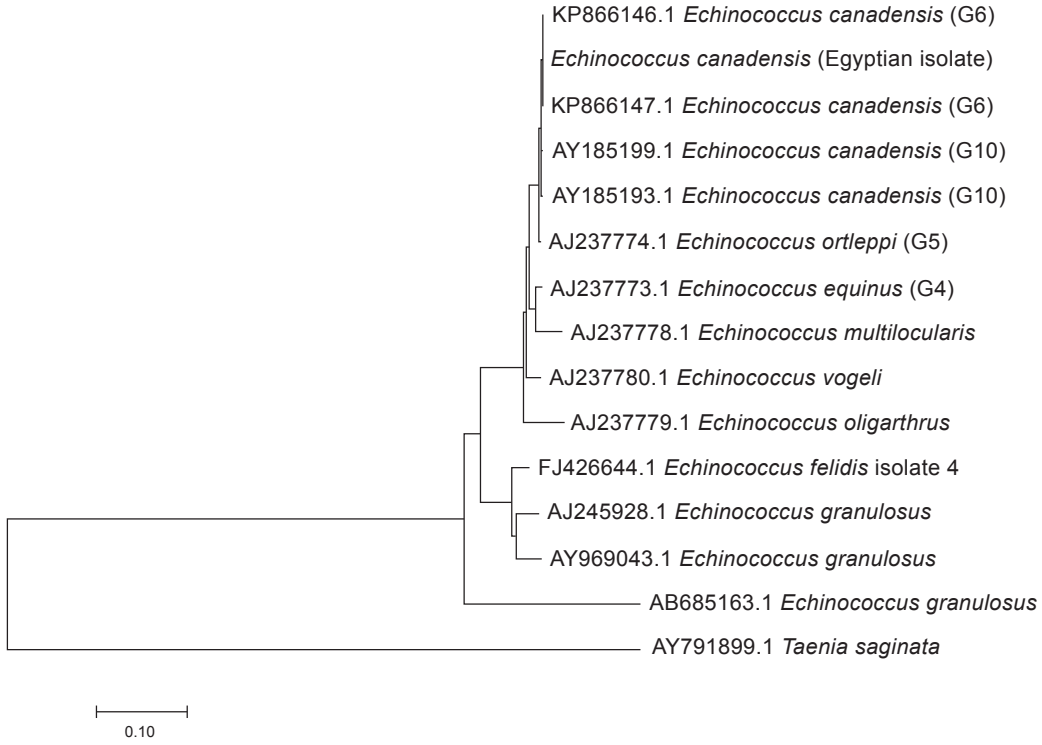

Fig. 4. Genetic relationship between an *E. canadensis* isolate from a camel HC (accession no.: MK460272) and the reference sequences of genotypes G1–G8 based on phylogenetic analysis of ITS1 nucleotide sequences.

was performed using the Basic Local Alignment Search Tool (BLAST, blast.ncbi.nlm.nih.gov). The obtained DNA sequences were subsequently aligned and compared with verified sequences of *E. granulosus* strains available in GenBank using ClustalW and Bioedit software. The phylogenetic tree was constructed using the software program MEGA 7. The sequences analysed in the present study were finally deposited in GenBank.

Ethical Approval and/or Informed Consent

All animal research reported in the present study were followed animal care and handling were carried out accordance with the institutional guidelines of Cairo University, Egypt.

Results

Results of conventional PCR

All DNA isolates (from 3 camel, one cattle, one sheep, 3 donkey and 3 rabbit secondary HCs were amplified to obtain bands of the same molecular size of 1115 bp, which is positive for hydatid species (Fig. 1).

Sequencing of PCR products

Sequencing of the ITS1 gene was performed to identify the *E. granulosus* genotypes. The partial nucleotide sequences of the ITS1 gene of the eleven isolates (3 camels, 1 cattle, 1 sheep, 3 donkeys and 3 rabbits) were aligned with the reference sequences of the genotypes G1–G8 using BLAST search. All sequences were compared with the identified sequences of *Echinococcus* species using soft ware program (MEGA7) for designing the phylogenetic trees, which confirmed the preliminary results obtained by BLAST search.

The three camel HCs produced sequences as G6; camel strains AJ237775.1, AJ237775.1 and KP866147.1 that shared 95, 97 and 97 % homology respectively, with the reference partial nucleotide sequence of *E. canadensis*. The GenBank sequence accession numbers of the 3 isolates were MK460268, MK460269 and MK460272 (Table 1, Figs. 2, 3 and 4.)

In the case of cattle, the HC isolate produced a sequence that shared 99 % identity with the reference partial nucleotide sequence of *E. granulosus s. s.* (G1, sheep strain AY969044.1). The GenBank sequence accession number was MK460273 (Table 1 and Fig. 5).

Additionally, the sheep isolate produced a sequence that shared 99 % homology with the reference partial nucleotide sequence of *E. granulosus s. s.* (G1, sheep strain KJ363926.1). The GenBank sequence accession number was MK460270 (Table 1 and Fig. 6).

The three donkey HC isolates produced sequences that shared 97 % homology with the reference partial nucleotide sequence of *E. equinus* (G4, horse strain AJ237773.1). The GenBank sequence accession number was MK460266 (Table 1 and Fig. 7)).

Moreover, two of the three rabbit secondary HCs isolates exhibited a sequence that shared 96 % homology with the reference

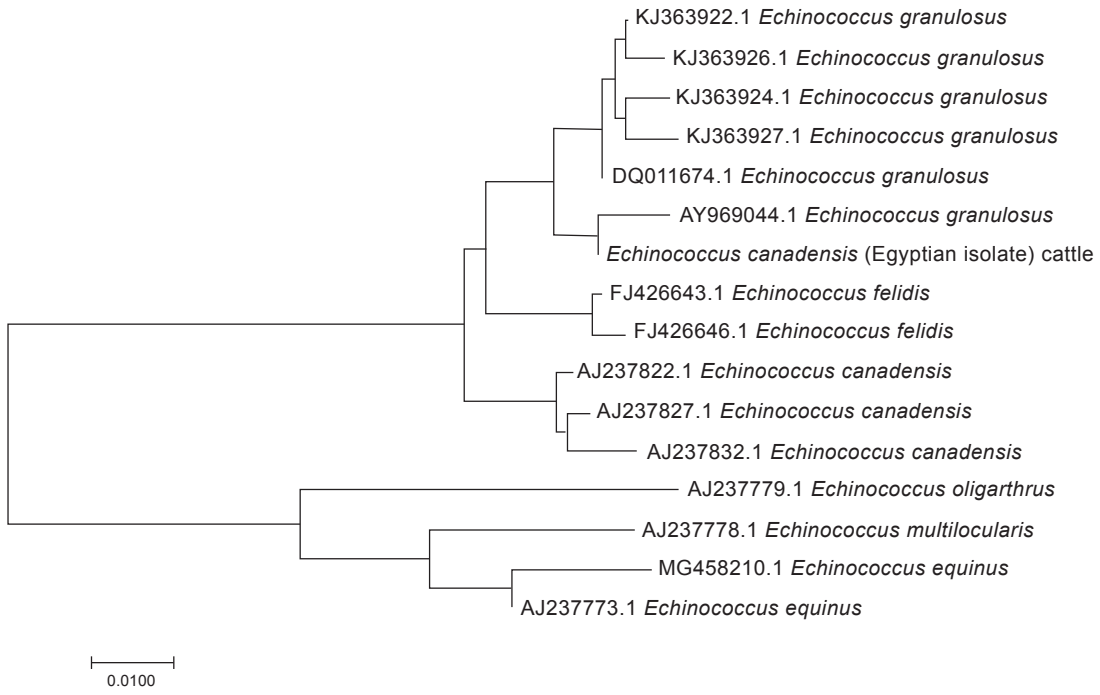

Fig. 5. Genetic relationship between an *E. granulosus s. s.* isolate from a cattle HC (accession no.: MK460273) and the reference sequences of genotypes G1–G8 based on phylogenetic analysis of ITS1 nucleotide sequences.

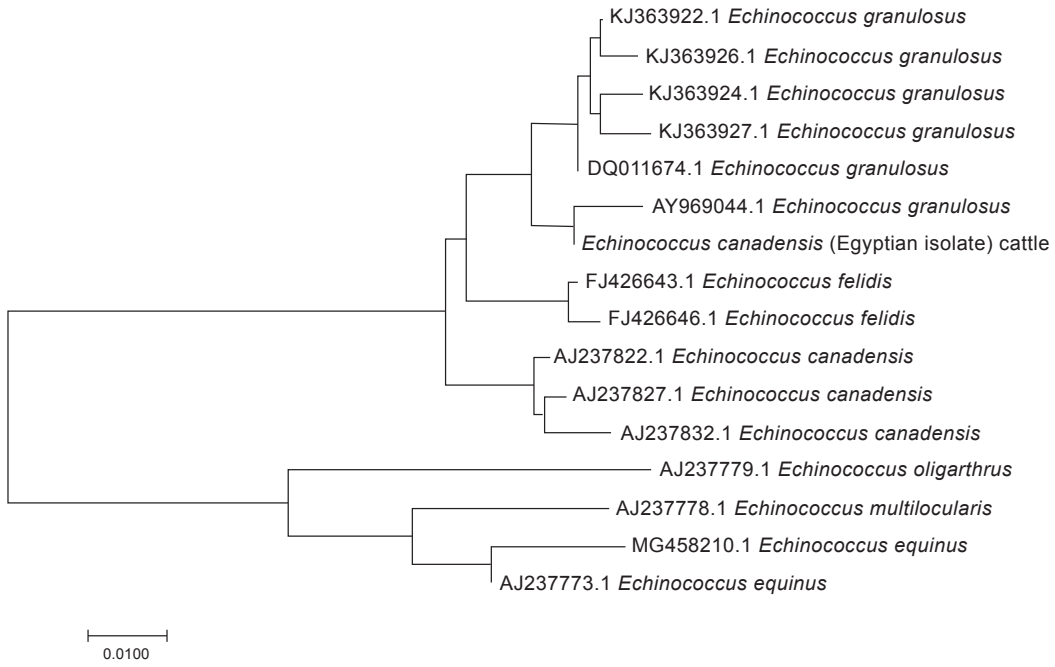

Fig. 6. Genetic relationship between an *E. granulosus s. s.* isolate from a sheep HC (accession no.: MK460270) and the reference sequences of genotypes G1–G8 based on phylogenetic analysis of ITS1 nucleotide sequences.

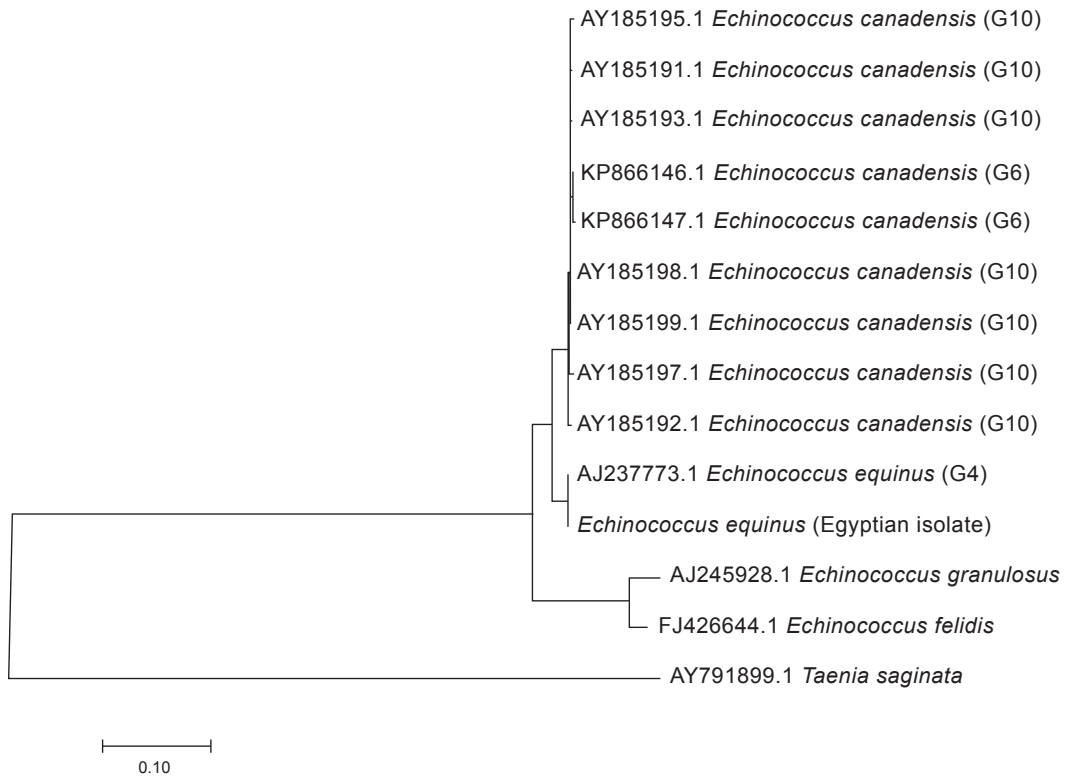

Fig. 7. Genetic relationship between *E. equinus* isolates from donkeys (accession no.: MK460266) and the reference sequences of genotypes G1–G8 based on phylogenetic analysis of ITS1 nucleotide sequences.

partial nucleotide sequence of *E. canadensis* (G6, camel strain AJ237775.1), while the third sequence exhibited 98 % homology with the reference partial nucleotide sequence of *E. canadensis* (G6, camel strain KP866147.1). The GenBank sequence accession numbers were MK460267 and MK460271 (Table 1, Figs. 8, 9).

Detection of the presence of genetic mutation during parasite transmission between two intermediate hosts

Partial nucleotide sequences of the ITS1 gene of rabbit secondary HCs and camel HCs were aligned and compared with those of the G6 genotype (camel strain) in GenBank. Both camel HCs and rabbit secondary HCs showed the same sequence identity matrix (0.216), which indicated the absence of mutation. However, a single nucleotide substitution of adenine to guanine at position 47 (A47G) was observed in the rabbit secondary HCs sequence, as shown in Fig. 10. The camel HCs and rabbit secondary HCs partial ITS1 gene nucleotide sequences were registered in GenBank (MK460272 and MK460271, respectively).

Discussion

CE is a cyclo-zoonotic disease that is distributed worldwide (Ahmadi, 2005). The first step in the prevention and clearance of infections from a specific area is identification of the strain(s) responsible for dissemination of infection and determination of the life cycle of the strain (Abdel Aziz *et al*., 2016). Therefore, in this study, the collected HCs were subjected to molecular characterization. Amplification of ITS1 by PCR showed a DNA band of the same molecular size of 1115 bp for all the isolates. This result was consistent with the results of Madawy *et al*., 2011 and Harandi *et al*., 2002, who found that the amplified DNA bands of the ITS1 gene had molecular sizes of 1115 bp in different HCs isolates (cattle and sheep in Egypt and sheep, goat, cattle and camel in Iran, respectively).

The partial nucleotide sequence of the ITS1 gene of the three isolates obtained from HCs camels belonged to *E. canadensis* (G6, camel strain). This result was consistent with the results of Khalifa *et al*., 2014 that studied a camel lung HCs in Egypt and identified G6 by gene sequencing of the NADH 1 gene. Abdel Aziz *et al*., 2016, found that 90.5 % of the HCs isolates collected from the lungs of slaughtered camels at the Toukh abattoir, Qalyubia Governorate, Egypt, were identified as *E. canadensis* (G6), and 9.5 % was identified as *E. ortleppi* (G5), which were from newly imported Sudanese camels. Amer *et al*., 2015, demonstrated the presence of *E. canadensis* (G6) in 26 out of 28 Egyptian camel HCs, while the 2 remaining cysts belonged to *E. granulosus s. s.* (G1) and *E. ortleppi* (G5). Additionally, in Sudan, Omer *et al.*, 2010, recorded the predominance of the camel strain (G6) and the infectivity of this strain towards humans. Moreover, Abushhewa *et al*., 2010, detected G6 in camels in Libya, and Harandi *et al*., 2002 and Hosseinzadeh *et al*., 2012, detected the camel strain in Iranian camels.

The circulation of the camel strain (G6) in camels suggests that specific mechanisms are responsible for the persistence of this

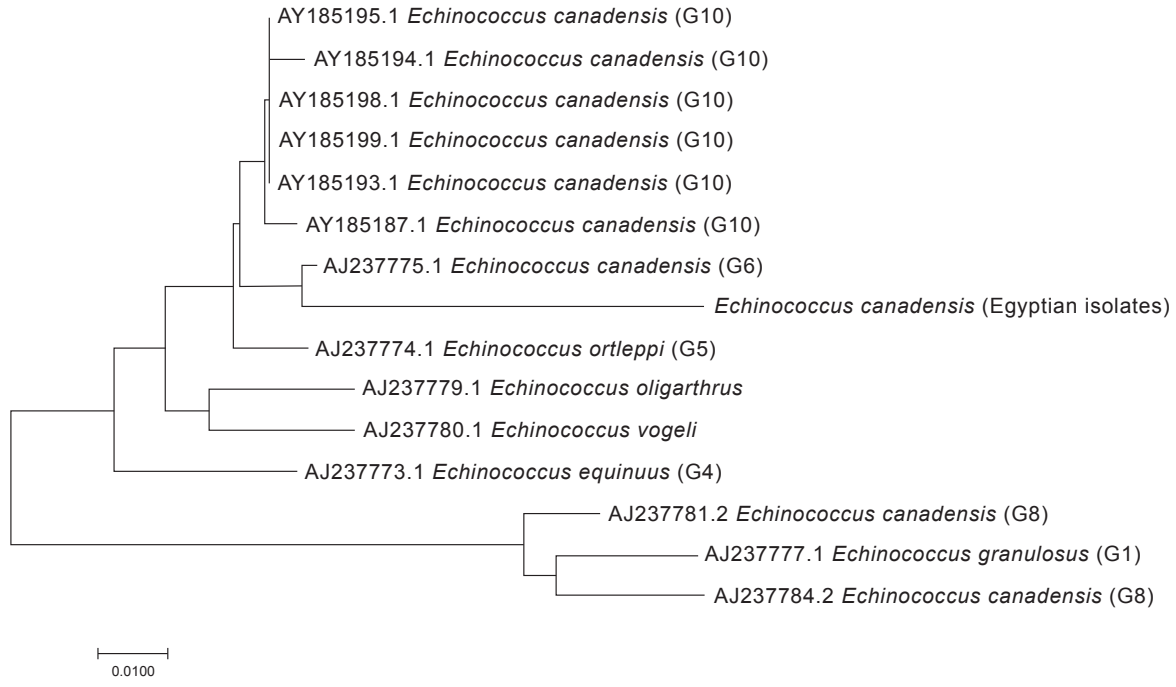

Fig. 8. Genetic relationship between an *E. canadensis* isolate from HCs of experimentally infected rabbits (accession no.: MK460267) and the reference sequences of genotypes G1–G8 based on phylogenetic analysis of ITS1 nucleotide sequences.

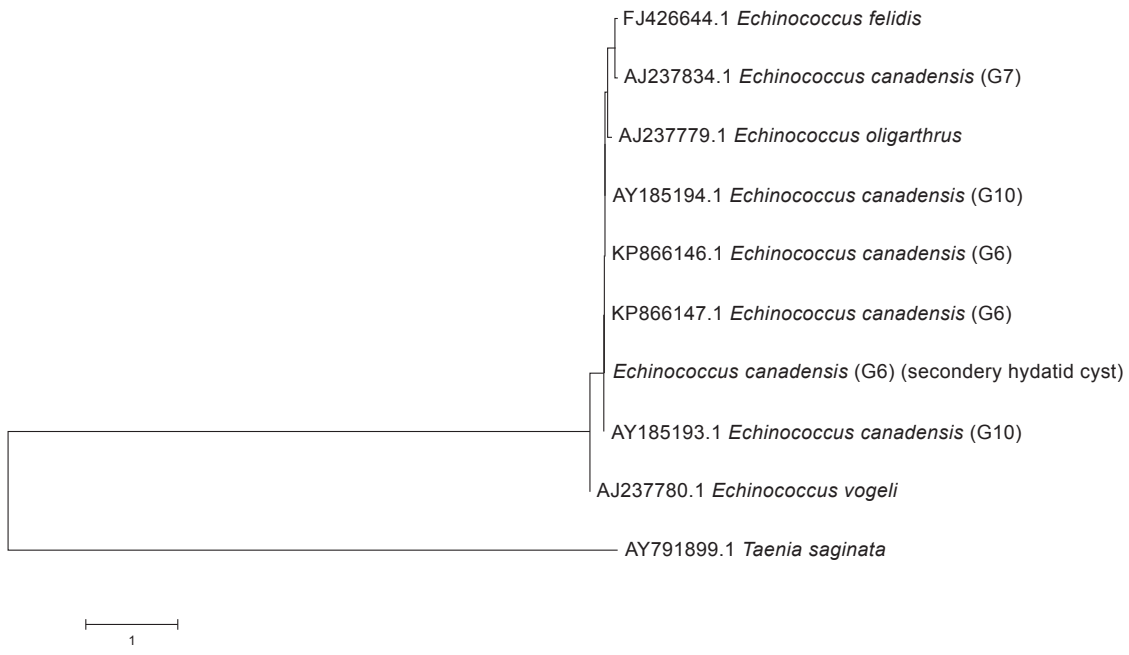

Fig. 9. Genetic relationship between an *E. canadensis* isolate from HCs of experimentally infected rabbits (accession no.: MK460271) and the reference sequences of genotypes G1–G8 based on phylogenetic analysis of ITS1 nucleotide sequences.

strain in this area. This effect is probably due to close relationships between dogs and camels in the study area (Dinkel *et al*., 2004). In poor communities, the slaughtering process is performed in open spaces, so dogs can be easily fed the viscera of slaughtered animals carrying the HCs strains, which then complete their life cycles and become adults inside the intestine of the dogs (Abdel Aziz *et al*., 2016).

The HCs isolates of cattle and sheep were identical to *E. granulosus s. s.* (G1, sheep strain). This result was consistent with the results of Madawy *et al*., 2011 and Kandil *et al*., 2016 who detected the sheep strain (G1) in both cattle and sheep in Egypt. Amer *et al*., 2015, identified that G1 and G6 from sheep in Egypt. Abbas *et al*., 2016, detected *E. granulosus s. s.* (G1) and *E. ortleppi* (G5) from cattle slaughtered at the Mansoura abattoir, Egypt. So, G1 is the most common genotype in grazing animals (cattle and sheep) due to its direct contact with dogs. In Iran, Jamali *et al*., 2004 and Parsa *et al*., 2011, identified sheep and cow isolates as sheep strains, and Hosseinzadeh *et al*., 2012, identified G1 in sheep and G6/7 in cattle**.** Bardonnet *et al*., 2003 and Busi *et al*., 2006, identified the sheep strain (G1) in cattle and sheep in North Algeria and Italy, respectively. In Pakistan, Ali *et al*., 2015, found that the *E. granulosus s. s.* (G1) and G6 genotypes were responsible for cattle infections. Also, Barghash *et al*., 2017, recorded the existence of two main genotypes; G6 (camel strain) followed by G1 (Sheep strain) in the majority of the studied human and animal isolates (camels, cattle, buffaloes, sheep, goats, pigs and donkeys) from different regions of Egypt. While, Sofi *et al*., 2019, recorded that G6 genotype of C.E. in buffaloes was the first report in the Northern India. Also, in Nigeria, Ohiolei *et al*., 2019, recorded that *E. canadensis* (G6/G7) were the isolate of cattle which showed 99 – 100% identical to previously reported G6/G7 haplotypes across Europe, Asia, North and East Africa.

The three examined isolates from donkey HCs belonged to *E. equinus* (G4, horse strain). This result was consistent with the results of (Aboelhadid *et al*., 2013), who found that HC isolates from donkeys in Beni-Suef, Egypt, belonged to *E. equinus*. In Southern Germany, Blutke *et al*., 2010, identified the genotypes of HCs isolates from mare lungs to belong to *E. equinus*.

Donkeys with *E. equinus*-containing HCs may act as sources of infections for dogs and foxes in Egypt via unhygienic disposal of donkey carcasses near water canals or cultivable land, making the carcasses easily accessible to stray dogs or foxes. Additionally, feeding of donkeys to carnivores in zoos may cause parasite transmission. Both pathways play an important role in the maintenance of the *Echinococcus* species life cycle, making the carcasses of these infected equines, especially donkeys, major sources of infection for dogs and foxes (Aboelhadid *et al*., 2013). Therefore, post-mortem examination of donkeys before use as food for wild animals, hygienic disposal of the carcasses of these animals and proper destruction of the infected offal are recommended.

The rabbit secondary HCs isolates belonged to *E. canadensis* (G6, camel strain). Partial nucleotide sequences of the ITS1 gene of rabbit secondary HCs isolates and camel HCs isolates were aligned and compared with those of the G6 genotype (camel strain) in GenBank. Both the camel HCs and rabbit secondary HCs showed the same sequence identity matrix, indicating the absence

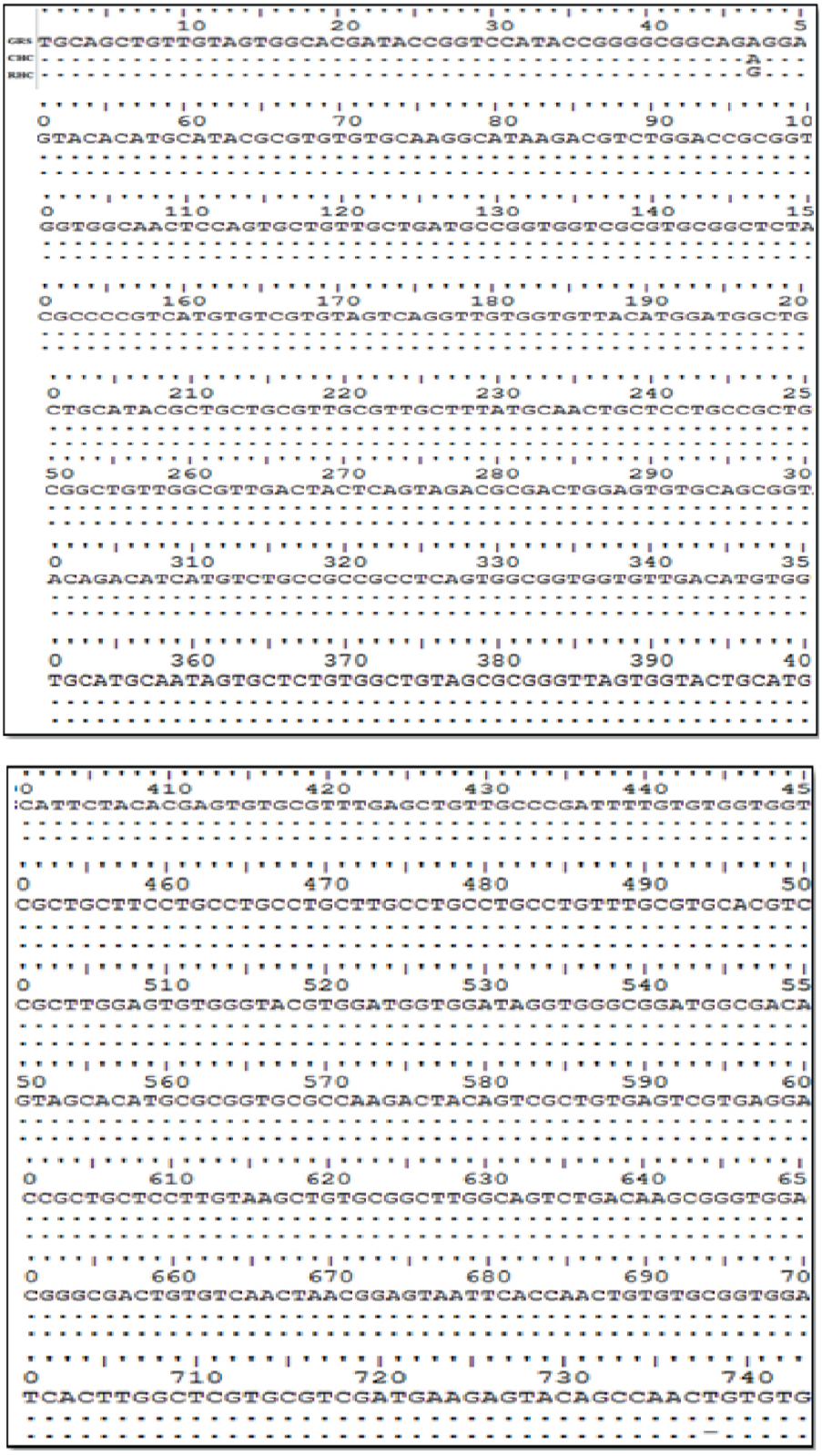

Fig. 10. Position of nucleotide substitution in the rabbit HC partial ITS1 nucleotide sequence.
GRS: GenBank reference sequence; CHC: camel HC; RHC: rabbit HC.

of mutation in the rabbit secondary HCs. Only a single nucleotide substitution of adenine to guanine at position 47 (A47G) occurred in the rabbit secondary HCs sequence. M'rad *et al*., 2010, recorded a single nucleotide substitution (C44T) in one bovine isolate compared to the G3 genotype in GenBank and explained that this substitution represents either a genotypic variant or a distinct strain that resembles the G3 genotype. Therefore, this aspect requires further investigation.

According to the results of our study, the *E. granulosus* genotype is not mainly host dependent. The same genotype was isolated from different intermediate hosts. In this study, G1 was collected from both cattle and sheep. In addition, G6 exhibited no changes when transmitted from camel to rabbit.

Conflict of Interest

Authors state no conflict of interest.

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