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Genetic Diversity of *Echinococcus granlosus* isolated from farm animals by using nuclear and mitochondrial genetic loci.

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Abstract : Determination of the genotypes *of Echinococcus granulosus* (*E.granulosus*) in farm animals of Egypt and Italy is the purpose of our study. Our endeavor describe the rapid diagnosis and characterization of *E. granulosus* genotypes by a specific and sensitive PCR, semi nested PCR system. Characterization of genotypes G1 for sheep , goats and cattle while G6 for camel. The Partial nucleotide sequences (570 pb) of CO1 and ND1 (600 bp) of the *E. granulosus* obtained from sheep, goats, cattle and camel were aligned with the reference sequences of the genotype; G1- G6. All the examined isolates products sequences are variable homologous to the other nucleotide sequence of *E. granulosus* isolates from different countries. GeneBank sequences accession No. for COX1 are KX379147, KX379146, KX379145 and KX 379148 while accession NO. for ND1 are KX298250, KX298249, KX298248 and KX379144 of cattle, sheep, goat and camel respectively. Current resulted data of the present attempt indicate some epidemiological features and molecular characteristics of *E. granulosus* in Egyptian and Italian farm animals.
Keywords: Genetic , Echinococcus, nuclear , mitochondrial and genotype.

Introduction

Cystic echinococcosis is a standout amongst the most common parasitic diseases of domesticated and wild animals, and an impressive reason for dreariness and mortality all over the world [1]. Cystic echinococcosis , caused by the metacestode of the dog tape worm *Echinococcus species*. It is a worldwide zoonotic disease which is financially imperative and constitutes a heart to general public health in numerous countries [2]. Parasitic taxonomy and phylogenetic tree of the genus Echinococcus have remained a dubious issue for quite a while [1]. Various *E. granulosus* strains, designated G1to G10 have been perceived[3]. These particular genotypes incorporate G1 and G2 as sheep strains. While, G3 and G5 as bovine strains, as well G4 and G6 as camel and horse strains[4]. Current taxonomic reviews have suggested *E. granulosus* in 4 distinct groups, including *E. granulosus sensustricto*(G1-G3 genotypes), *E. equinus* (G4), *E. ortleppi*(G5), and *E. canadensis* (G6-G10) [5].These genotypes have a wide geographical distribution, demonstrate low transitional host specificity [6]. More as of late, the hereditary diverisity in the mt DNA of *E. granulosus* has been accounted for to be higher than already accepted[7]. The genetic variation of *E. species* can reflect contrasts in infectivity for specific host species. Hence it is of huge significance to phylogenetically portray *E. granulosus* population structure [8]. Useful genetic markers in taxonomic studies as mt DNA because it is haploid, multicopy, non-recombining and maternally inherited [9]. Besides, cytochrome c oxidase subunit 1 (cox1) gene as a mitochondrial gene, it has been appeared to be a good candidate in the intraspecific genetic variability classification of the of *E.*

granulosus even short length DNA sequences[10]. For instance, [4] detected 43 mt DNA haplotypes in 181 Chinese isolates from different Chinese area in sequences of cox1 of *E. granulosus* . A degree of genetic hetrologus between *E. granulosus* isolates is one of this parasites features [11]. It is essential that such studies are completed, as hereditary assorted qualities may reflect contrasts in infectivity, particularly to people, with vital ramifications for the study of disease transmission of hydatid sickness[12]. Furthermore, the marvel of strain variety is a critical thought later on configuration and improvement of antibodies, analytic reagents and medications successful against the Echincococcus living beings. Nonetheless, costs, time utilization and/or levels of DNA quality do as a rule not permit the use of these strategies for screening of large quantities of samples. for the configuration of reasonable and asset effective control programs-particularly in developing countries such information are earnestly required. In zones where creatures harbor different E. species or genotypes with various pathogenicity of human portraying the organic cycles and courses of transmission with pertinence to human infection is important[13]. The purpose of the present endeavor was to investigate the population genetic structure and the mitochondrial variability and of *E. granulosus* in farm animals different intermediate hosts. This outcomes will be key for epidemiological studies examining the science and transmission progression of these parasites, and will support research on the determination, control, and aversion of this disease.

Material and Methods

1- Cyst collection.

E. granulosus hydatid cysts were collected from lung and liver of infected sheep, goats, cattle and camel. Cysts were processed separately under sterile conditions. Cysts were classified morphologically as fertile, sterile (acephalocysts), or degenerated (calcified or caseous). Fertility was defined by the microscopic detection of protoscoleces (larvae). In this study, an isolates represents tissue of germinal laminar layer from sterile cyst collected from an individual hydatid cyst. Germinal layer tissue were rinsed in saline then stored at -20°C until DNA extraction[14].

2- DNA extraction:

DNA was isolated from ethanol preserved or frozen samples. Up to 0.5g of cyst tissue of germinal and laminar layers were cut into small pieces. DNA extraction kit (**P**urelink Genomic DNA minikit**, I**nvitrogen) according to the manufacturer's instructions. The extracted DNA was kept at −20°C until further analysis could be performed. DNA obtained was used as templates for polymerase chain reaction (PCR).

3-PCR assay specific for E. granulosusGenotypingand mitochondrial DNA sequencing:

PCR was performed in a 100µl volume containing 10 m M Tris –Hcl (Ph 8.3), 50 m MKCl, 2 m M MgCl2, 200 µM of each dNTP, 50 pmol of each primer (according to table 1) and 2.5 units ampli-tag polymerase (Perkin Elmer Biosystems). Amplification was carried out for 40 cycles as follows: denaturation for 30 s at 94°C, annealing for 1 min at (according to Table 1) and elongation for 40 s at 72°C. Semi-nested PCRS specific for G6/7 camel. The First G6/7 amplicon was used in a second step as a DNA templet.

PCR for sequencing the mitochondrial genes NADH dehydrogenase I (NDI) and cytochrome coxidase subunit 1 (COI) as described by Bowles and McManus [15] and Bowles et al. [10]. After amplification, 10µl of the amplification products were detected on a 1.5% gel Ethidium bromide stained agarose gel.

Table 1: PCR primers and annealing temperatures used in E. granulosus Genotyping:

Primer name	Serotype	Primer sequence (5' to 3')	Expected size (bp)	Annealing Temperature (°C)
E.g.ss1 F	G1	GTA TTT TGT AAA GTT GTT CTA	254	57
E.g.ss1 R		CTA AAT CAC ATC ATC TTA CAA		
E.g.cs1 F	G6/7	ATT TTT AAA ATG TTC GTC CTG	254	53
E.g.cs1 R		CTA AAT AAT ATC ATA TTA CAA C		
E.g. camel. F	G6/7 camel	ATG GTC CAC CTA TTA TTT CA	171	60
E.g. camel. R		As E.g.cs1 R		
NDBT-F	NDI	GGT TTG TTG CAG AGG TTT	570	55
NDBT-R		TAA TCA AAT GGC GTA CGA T		
JB3 – F	COI	TTT TTT GGG CAT CCT GAG GTT TAT	600	55
JB4.5 – R		TAA AGA AAG AAC ATA ATG AAA ATG		

4- Analysis of PCR amplification products (amplicons):

The resulting PCR amplicons (10-15 μl) were analyzed by 1.5% agarose gel electrophoresis as described by Sambrook and Russell. [16]. The DNA bands were visualized using ultraviolet transillumination after gel staining with Ethidium bromide gel (0.7 μg/ml). The PCR amplicons of the proper predicted size respectively to each primers set.

5-phylogenetic analyses:

Direct sequencing of PCR amplicons: The PCR products were purified using Min Elute PCR Purification kit and directly sequenced in both directions with the same primers used to generate PCR amplicons. Sequencing was done in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems)system using the dideoxy chain-termination method, based on the incorporation of fluorescent-labeled dideoxynucleotide terminators.

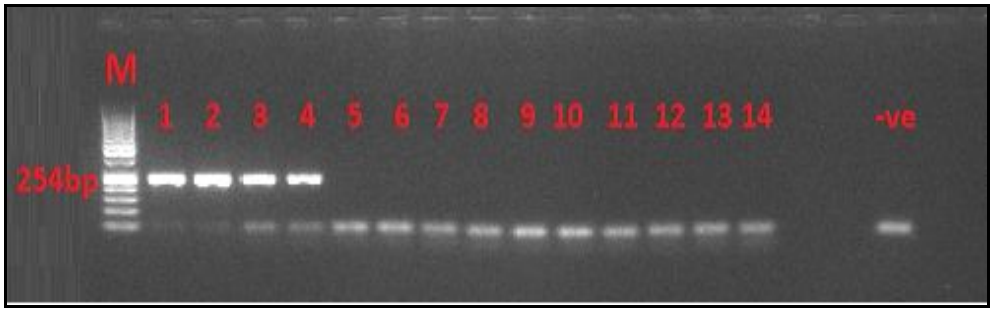
Nucleotide sequence analysis was undertaken using the National Center for Biotechnology Information BLAST programs and databases (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignments were made with the Clustal W method andBioedit software and compared with GeneBank sequences. Comparison of gene sequences with the identified homological sequences of *Echinococcus* species, were performed using the MEGA6 program [17]. Phylogenetic tree was constructed using the program Neighbor- joining in the same software.

Results

Detection of E. granulosus DNA in all cyst samples from different hosts by PCR, which taq polymerase in presence of specific primers amplified the target sequence from all cestode species and different genotypes which were tested. The G1 PCR selectively amplified the G1 genotype of E. granulosus with a specific band of 254 bp from sheep, goat and cattle samples.

The G65/6/7PCR genotypes of E. granulosus with a specific band of 254bp fragment were also confirmed by sequencing of the G6 PCR amplicon. Discrimination between E. granulosus G6 and G7 , the amplification of the PCR product underwent specific semi nested PCR. Both PCRs resulted in a specific product of 171 bp.

Additional gene sequencing of mitochondrial CO1 and ND1 was performedTo identify the Echinococcus species . Sheep, goats and cattle origin samples were shown to belong to E. granulosus G1 while only camel strain is belong to G6 group.



Fig(1): Amplification products from *E. granulosus* with PCR resolved on a 1.5% agarose gel . PCR products were specific for G1. Lane (1-4)) *E. granolosus* from sheep and goat origin .Lanes: (M) 50 bp DNA ladder (consists of repeats of 50 bp fragment size),(Fermintas).

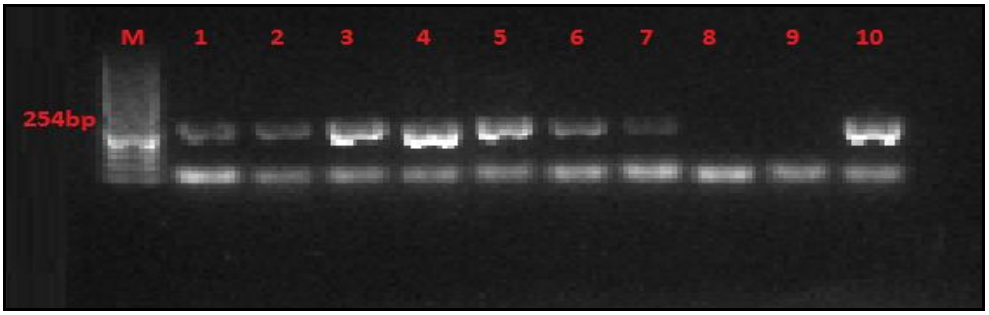
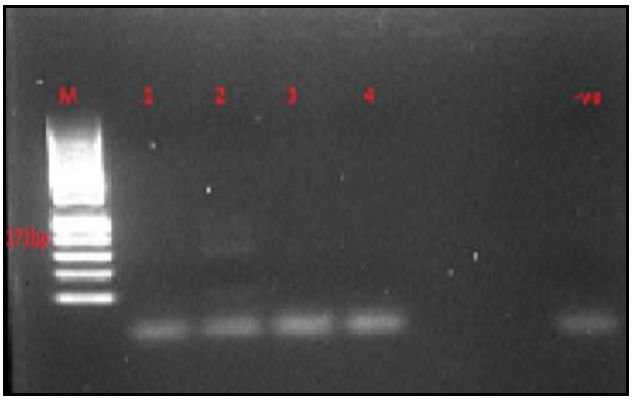


Fig (2): Amplification products of *E. granulosus* from three different hosts (sheep, goat and cattle) with PCR resolved on 1.5% agarose gel. PCR products were G1, Lanes: (M) 50 bp DNA ladder (consists of repeats of 50 bp fragment size, (Fermintas).



Fig(3): Semi nested PCR: Amplification products of *E. granulosus* from camel origin with PCR resolved on 1.5% agarose gel. PCR products were *E.granulosus*G6. Lanes: (M) 50 bp DNA ladder (consists of repeats of 50 bp fragment size, (Fermintas).

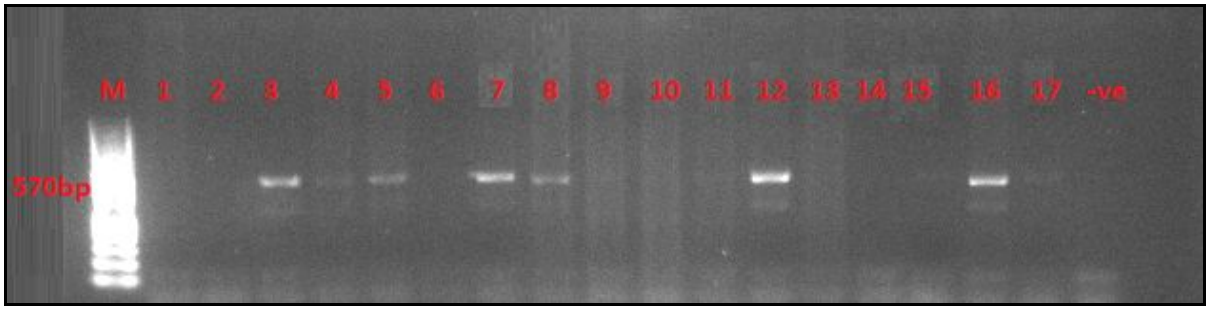


Fig (4): PCR results of *E. granulosus* template DNA using mitochondrial C oxidase subunit 1 gene . Lane (3, 4, 5, 7, 8, 12, 16) denote template DNA isolated from germinal layer of hydatedcyst.Lanes: (M) 50 bp DNA ladder (consists of repeats of 50 bp fragment size),(Fermintas).

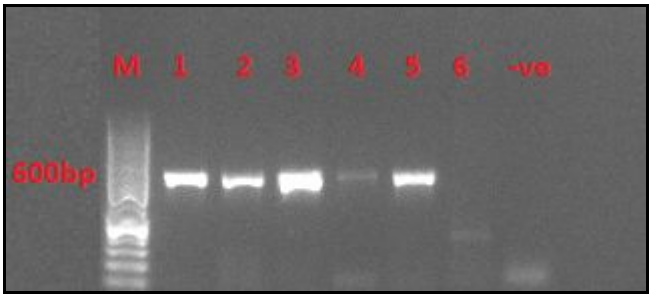
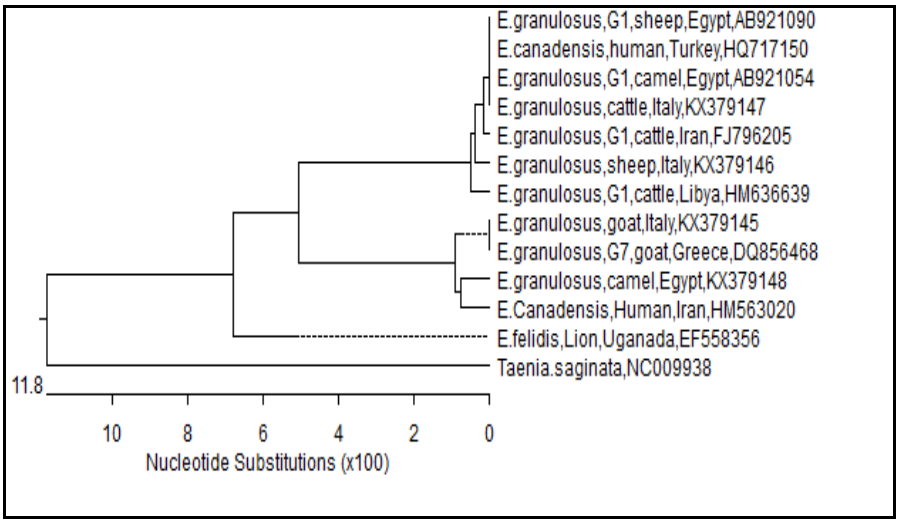
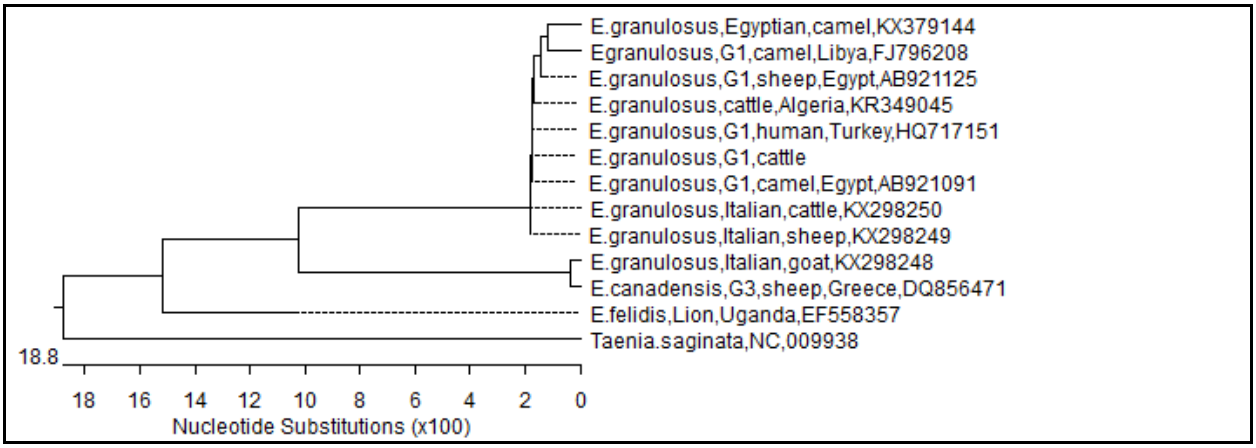


Fig (5): PCR results of *E. granulosus* template DNA using mitochondrial NADH dehydrogenase 1 gene. Lanes (1-6) denote template DNA isolated from germinal layer of hydatid cyst.Lanes: (M) 50 bp DNA ladder (consists of repeats of 50 bp fragment size),(Fermintas).



Fig(6). Phylogenetic relationships of E. granulosus isolates according to CO1 sequences in correlation with different hosts.



Fig(7). Phylogenetic relationships of E. granulosus isolates according to ND1 sequences in correlation with different hosts.

The Partial nucleotide sequences (570 pb) of CO1 and ND1 (600 bp) of the *E.granulosus* obtained from sheep, goats, cattle and camel were aligned with different country origin isolate sequences of the genotype; G1-G6 using BLAST search. All the examined Egyptian and Italian isolates product sequences having variable homology% to the other isolates of *E. granulosus* from different countries as shown in current two phylogenetic trees of *E. granulosus* .

Table 2. Echinococcus species and GenBank accession numbers of COX1 and NAD1 used in phylogenetic analysis .

E. granulosus	**Genotype classification**	**Intermediate host**	**Country**	**GenBank Accession No. for COX1**	**GenBank Accession No. for NAD1**
E. granulosus	G1	cattle	Egypt	KX379147	KX298250
E. granulosus	G1	sheep	Italy	KX379146	KX298249
E. granulosus	G1	goat	Italy	KX379145	KX298248
E. granulosus	G6	camel	Egypt	KX379148	KX379144

Discussion:

Echinococcus granulosus is an important pathogenic parasite all over the world, involved with hydatidosis of human and mammals. This disease can bring about high mortality in people and in addition monetary misfortunes in animals. The dispersion of Echinococcus genotypes contrasts from geographic area to another and from host to host. To date, a few techniques have been utilized to decide the hereditary assorted qualities of E. granulosus. Late studies that have conducted the molecular identification of E. granulosus found that the G1 genotype is the main genotype in all over the world specially Middle East (Egypt) and Europe (Italy), and there are a small number of the G3, G6, and G7 genotypes [17]. In our study, 5 isolates were identified as the E. granulosus G1 genotype (from Egypt and Italy), while two isolates (both derived from Camel in Egypt) belonged to the G6 genotype. The two genotypes on this study contribute the part of common Echinococcus genotypes in Egypt and Italy due to presence of dogs near to cattle, sheep, goat and camel farms who are usually straying on streets and near abattoirs, feeding on offal of slaughtered animals or carcasses of dead animals in rural areas. Defiling the environment with Echinococcus eggs due to stray canines additionally have free access to yards and fields of local animals. Our outcome results are in part concur with [18], who demonstrated that three Echinococcus species are available in homestead animals in Egypt. These species are E. granulosus -sheep genotype or (G1), E. canadensis - camel genotype or (G6), and E. ortleppi - cattle genotype or (G5). Interestingly, [19] identified and asserted that G1 was basic in people, camels and sheep hosts by using strain specific PCR. Interestingly in Libya, G1 is the restrictive genotype in cattle, while G6 overwhelms in camels [7]. G1 is likewise the regular genotype in various hosts in Ethiopia [20], Palestine [5], Iran [21], India [22], China [23] and Mongolia [24]. Echinococcus granulosus (G1,G2,G3 complex) is likewise the real genotype in cattle, sheep and goats in numerous European Nations [11,25,26].

Increasingly molecular studies and data gathered in recent years shows that at least three species of the *E. granulosus* complex are circulating in domestic animals in Africa . These include *E. granulosus* (G1,G2,G3 genotypes), *E. ortleppi* (G5 genotype, cattle strain) and *E. Canadensis* (G6–G10 genotypes). *E. granulosus* and *E. canadensis* have both been identified in African countries like Tunisia, Algeria, Libya, Kenya and Ethiopia. *E. Canadensis* is so far only Species found in Mauritania and Egypt. By domesticated animals species, Sheep have all the earmarks of being solely contaminated by E. granulosus in every one of the nations where ovine CE has been accounted for aside from Sudan, with the G1 variation representing 90% of the segregates and the G2 genotype for the remaining. Not surprisingly, E. granulosus is preferred adjusted to sheep over E. Canadensis [20,27].

Molecular studies in goats are limited so far to Kenya and Sudan. African goats are prevalently contaminated by *E. granulosus* and *E. canadensis* representing the rest of the contaminations. Significantly, the sheep–dog cycle assumes a basic part in zoonotic cycle of the disease to people

Three Echinococcus species have been found to infect cattle in Africa, including *E. granulosus*, *E. ortleppi* and *E. canadensis*. The E. granulosus G1 variation is dominating in separates from Algeria, Ethiopia and Tunisia, with genotype frequencies running from 86% to 100%. To some degree conflicting results have been acquired in Kenya and Libya, with some overviews reporting E. granulosus G1 frequencies running from 97% to 100% [28,29], while others found that E. canadensis G6–G7 were the genotypes coursing most oftentimes in steers from these nations [7,13]. Interestingly, *E. ortleppi* has not been identified in cattle out of Kenya and Sudan so far.

Camels are infected by both E. granulosus or E. Canadensis G6–G7 with variable genotype incidences in Algeria, Ethiopia, Kenya, Libya and Tunisia, while E. canadensis G6–G7 is the main genotype discovered contaminating camels in Egypt, Mauritania and Sudan. These genotypes can also successfully infect sheep, goats and cattle [30,31]. On the contrary, camels appear to be suitable hosts for *E. granulosus* G1 infections [32]. Taking together, the features give solid confirmation that camels assume an imperative part for the support of the Echinococcus life cycle in numerous parts of Africa.

A generous measure of genotyping information is right now accessible from the larger part of the European nations influenced by CE, permitting a fairly finish picture of the molecular differing qualities and geological conveyance of Echinococcus contaminations underway different animal hosts. A minimum four Echinococcus animal varieties have been recorded to circle in Europe. The accompanying species are available in Europe: E. multilocularis, E. granulosus (G1), E. ortleppi (G5) and E. equinus (G4). which, around the world, comprises of the strains G6 to G10. Romania *E. granulosus* was diagnosed in sheep, cattle [25]. Until the 1990s, the known extent was limited to central Europe. Today the parasite is isolated and characterized from Denmark, Netherlands, Belgium, France, Poland, Slovakia, Hungary and northern Italy [33,34,35].

Notably, *E. granulosus* G1 is the only genotype found in all the European isolates analyzed from intermediate and final exotic hosts to date [6,36]. These findings determine the main role of the dog-sheep cycle in the transmission of the parasite in Europe.

The genotyping of Egyptian and Italian isolates of E. granulosus from sheep, goat, cattle, and camel revealed the existence of 2 distinct strains (cattle-dog and camel-dog strains) by mtDNA markers [37]. As molecular analyses of the NADH dehydrogenase subunit 1 (ND1) and COX1 sequences were used to characterize deer isolates of E. granulosus from Canada and Finland, respectively [38,39]. The G1 PCR selectively amplified the G1 genotype of E. granulosus with a specific band of 254 bp. The G65/6/7PCR genotypes of E. granulosus with a characteristic band of 254bp fragment were also confirmed by sequencing of the G6. To discriminate between E. granulosus G6-G7 , the amplification of the product underwent different semi nested PCR. Both PCRs resulted in a specific product of 171 bp.

In brief, our study provides the fundamental information about genetic diversity analysis of a Egyptian and Italian isolates from different hosts so as to comprehend in subtle element the hereditary structure of E. granulosus populaces and transmission progression of echinococcosis in these locales. Our study information could strengthen disease surveillance in these regions and improve preventive measures, in addition to developing a strong control strategy.

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