Original Article

Algorithms based on PCR-RFLP of *nad1* gene for genotyping of *Echinococcus granulosus* from human and animal isolates in Egypt

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ABSTRACT

Background: Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is a simple rapid method for genotyping of *Echinococcus garnulosus sensu lato* (*E. granulosus s. l.*) in developing countries. Construction of algorithms based on PCR-RFLP using two restriction enzymes would be useful to study the genetic diversity of the parasite and would help in differentiation between ambiguous genotypes.

Objective: The goal of the present work was to develop algorithms based on RFLP of nicotinamide adenine dinucleotide dehydrogenase (NADH) subunit 1 (*nad1*) sequences of reference genotypes of *E. granulosus* retrieved from GenBank for genotyping of human and animal isolates of *E. granulosus* in Egypt.

Subjects and Methods: Retrieved *nad1* sequences of reference genotypes were digested *in silico* individually with two restriction enzymes; *Haemophilus influenza* (*HinfI*) and *Haemophilus aegyptius* (*HaeIII*). The constructed PCR-RFLP algorithms were used for genotyping of 50 human and animal isolates (19 human, 23 camels and 8 pigs) analyzed by PCR-RFLP. To confirm the validity PCR-RFLP algorithms, samples corresponding to determined and undetermined genotypes as inferred from the algorithms were sequenced.

Results: Utilizing PCR-RFLP and sequencing revealed that except for two cases (12.5%) which were typed as G1 among humans and one case as G5 in pigs (12.5%), G6 was the commonest genotype among human, camel and pig isolates collected.

Conclusion: The algorithms based on PCR-RFLP of *nad1* are valuable tools for genotyping of *E. granulosus s. l.* especially with *HinfI* RFLP algorithm. Sequencing is still needed to reveal the genotypes of undetermined or ambiguous isolates.

Keywords: algorithm, *E. granulosus*, Egypt, genotyping, *nad1*, PCR-RFLP.

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INTRODUCTION

Understanding the epidemiology of echinococcosis is troublesome in many endemic areas where the role of different host species involved in transmission needs clarification[1]. A major contribution in understanding the extensive genetic and phenotypic variation exhibited within the genus *Echinococcus* has been made through PCR-based approaches and sequencing of both nuclear and mitochondrial DNA. This resulted in clarification of transmission patterns and the role of different hosts species involved in the life cycle[2,3]. Notably, *E. granulosus* mitochondrial genes, especially *nad1* and *cox1* were considered as well-established vital molecular biomarkers for classifying genetic diversions of *E. granulosus* from human, cattle, sheep, and different animal species[4]. By molecular classification mitochondrial genes implied that *E. granulosus s. l.* is a species complex consisting of *E. granulosus sensu stricto* (genotypes G1-3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6-7, G8 and G10), and *E. felidis*[5,6]. Worldwide, genotype G1 proved to be the most prevalent[5,7,8], possibly due to its wide range of intermediate hosts facilitating higher spread within the environment[6,8,9]. It is worth mentioning that PCR-RFLP is a simple and rapid method for genotyping of *E. granulosus*, it is suitable for application in developing countries, where the disease is mostly prevalent[10].

Previously, we used PCR-RFLP targeting *nad1* gene for molecular characterization of fifty *E. granulosus* human and animal isolates from Egypt using the *HinfI* restriction endonuclease enzyme. Two RFLP patterns were obtained: pattern I in 95.2% of samples (12 human, 21 camel and 7 pig samples) with three fragments of 115, 218, and 738 bp; and pattern II in 4.8 % (2 human samples) with two fragments of 1035 and 36 bp. Four samples were not digested with *HinfI*. In total, 85.7% of human and 100% of camel

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and pig samples shared the same digestion pattern I, while pattern II appeared exclusively in two human cases (14.3%) out of the 14 typed[11]. However, the application of PCR-RFLP patterns is not broadly used as a result of heterogeneity of *Echinococcus* genome within different regions of the world. Consequently, designing and conducting a standardized pattern should be interpreted indigenously, to avoid unexpected mutations (such as nucleotide change/insertion or deletion) within the parasite genome[10]. Furthermore, a practical algorithm using more than one restriction enzyme should be constructed for interpretation of PCR-RFLP results[12].

In silico computer-based methods are rapid techniques widely used nowadays for genotyping of microorganisms, that are accurate and less costly[13]. They are facilitated by the great plethora of partial and complete sequences deposited in gene banks from wide geographical areas, as well as the availability of large numbers of analytical software. *In silico* PCR-RFLP was used for genotyping of several parasites like *T. vaginalis*[14], and *Leishmania* spp.[15] Some *in silico* studies were developed for genotyping of *Echinococcus* spp., where gene sequences retrieved from GenBank were cut with specific restriction enzymes to create a virtual RFLP pattern for genotypic differentiation[10,16].

Retrieval of the sequences of *nad1* gene of *E. granulosus* reference genotypes from GenBank, and their *in silico* digestion with restriction enzymes using RFLP software would allow the building of practical algorithms for genotyping of *E. granulosus* isolates using the expected obtained digestion patterns. Hence, the aims of the present study were to build practical algorithms based on *in silico* digestion of sequences of mitochondrial *nad1* gene of reference genotypes of *E. granulosus* by two commonly used restriction enzymes, *HinfI* and *HaeIII*. The constructed algorithms will be used for genotyping of Egyptian isolates of *E. granulosus* using PCR-RFLP. Subsequently, the results inferred from PCR-RFLP will be confirmed by DNA sequencing of selected samples representing the different RFLP patterns.

SUBJECTS AND METHODS

The present descriptive analytical study was conducted during the period from June 2018 to August 2019 at the Parasitology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

Study Design: The sequences of *nad1* gene of *E. granulosus* reference genotypes were digested with two commonly used restriction enzymes, *HinfI* and *HaeIII*, by means of *in silico* software program. The resulting restriction patterns were used for construction of practical algorithms to identify the different genotypes. Following DNA extraction from Egyptian human and animal isolates of *E. granulosus*, the *nad1* gene was amplified and digested with the individual restriction enzymes. The genotype of the isolates was determined from the constructed *in silico* algorithms. To confirm the validity PCR-RFLP algorithms, samples corresponding to determined and undetermined genotypes as inferred from the algorithms were sequenced.

Construction of algorithms based on RFLP of retrieved *nad1* gene of *E. granulosus* reference genotypes: Initially, the complete mitochondrial genome of *E. granulosus* G1 (AF297617) genotype was retrieved from the National Center for Biotechnology Information (NCBI) GenBank. The nucleotide sequence (1071-1078 bp) representing the *nad1* gene amplification region, according to Huttner *et al*.[17], was subsequently checked by applying the flanking primers on the complete mitogenome utilizing Primer-Map (www.bioinformatics.com). The deduced sequence was then aligned with the complete mitogenome of other genotypes "G3-G10" and *E. felidis* retrieved from the GenBank with the following accession numbers AF297617, KJ559023, AF346403, AB235846, AB208063, AB235847, AB235848, AB745463, and AB732958[18-20], utilizing the clustalW multiple alignment method within Geneious 10.1.3 software program. Inferred reference sequences were digested by *HinfI* and *HaeIII* restriction enzymes using "restriction sites" tool within Geneious 10.1.3 software program. Interpretation algorithms for the generated patterns were eventually constructed for genotyping of *E. granulosus* from human and animal isolates.

Samples' collection and parasitological examination: Fifty samples (19 humans, 23 camels and 8 pigs) that were used in our previous study[11] were included in the present study. Human samples were collected from Abdominal Ultrasonography Unit of Tropical Medicine Department, Kasr El-Aini Hospital, Cairo University, and from departments of Tropical Medicine, General Surgery and Cardiothoracic Surgery, Faculty of Medicine, Ain Shams University. Animal samples included 23 pulmonary camel and 8 hepatic pig cysts from condemned organs of camels and pigs slaughtered in Cairo Abattoir. Protoscolices from hydatid fluid samples were collected by centrifugation. For individual infertile cysts, the germinal layer was collected under aseptic conditions. Collected materials were washed three times with sterile saline solution and fixed in 95% ethanol until further molecular analysis.

DNA extraction and PCR amplification of *nad1* gene from human and animal samples: DNA extraction was done using "QIAamp® DNA Mini Kit" (Qiagen, Hilden, Germany) according to manufacturer's specifications. PCR amplification of a 1071-1078 bp fragment including the complete *nad1* gene was performed according to Huttner *et al*.[17] using a forward primer: 5´ TATTAAAAATATTGAGTTTGCGTC-3´ and a reverse

primer: 5´ TCTTGAAGTTAACAGCATCACGA T 3´, as previously described[11].

RFLP of the amplified *nad1* gene: *HinfI* and *HaeIII* restriction endonucleases were used individually to digest the purified PCR product of the *nad1* gene according to Chaâbane-Banaoues *et al*.[16]. Digestion was performed according to the manufacturer's instructions (Promega). *HinfI* cut through a five base palindromic restriction site "GANTC", while *HaeIII* cut through a four base palindromic restriction site "GGCC".

DNA sequencing and sequences analysis: Seven samples from 3 humans, 2 camels, and 2 pigs, represented the different genotypes deduced from the PCR-RFLP algorithms; and 4 samples from 1 human and 3 camels with undetermined genotypes according to the algorithms, were subjected to automated DNA sequencing based on Sagner technique[21]. Amplified PCR products were primarily purified using the QIAquick PCR purification kit according to the manufacturer's protocol. The BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Life Technologies, USA) was utilized. Acquired sequences were first edited to create consensus sequences and reviewed for their reliability using Geneious 10.1.3 software. Basic Local Alignment Search Tool (BLAST) algorithm was used for homology searches.

Ethical consideration: The research protocol was approved by the Ethics Committee of the Faculty of Medicine; Ain Shams University that complies with the 1964 Helsinki Declaration and regulations of the Egyptian Ministry of Higher Education. An oral consent from human subjects who participated in the study was obtained after a clear explanation of the study objectives. Patients with hydatid disease were already diagnosed and treated by surgery, PAIR combined with albendazole chemotherapy. Hydatid cysts were collected from slaughtered animals during inspection by veterinary officers at Cairo Abattoir, after approval from the authority of the slaughterhouse. No experiment was conducted on live animals.

RESULTS

Construction of algorithms based on RFLP of retrieved *nad1* gene of *E. granulosus s. l.* reference genotypes: Algorithms were constructed. using the *HinfI* and *HaeIII* restriction endonucleases. Table (1) and figures (1-3) demonstrate the numbers of fragments, digestion position from 5' end and size of fragments (bp). According to the algorithm, *HinfI*-RFLP of *nad1* gene had greater discriminatory power in genotyping of *E. granulosus s. l.* than *HaeIII*-RFLP. However, *HinfI* could not differentiate between G1-G3, or G6/G7, or G8 from *E. felidis*. Besides, *HaeIII* could not differentiate between G1-G3, or G5, G6/G7, G8, G10, or G4 from *E. felidis*. At the level of the two enzymes, all genotypes could be differentiated except for G1-G3 and G6/G7.

PCR-RFLP analysis of human and animal samples: Out of the 50 collected samples, successful amplification was obtained in 46 (92%) samples (16 humans, 22 camels, and 8 pigs) producing the expected 1069-1078 bp band on the agarose gel. Four (3 human and 1 camel) samples (8%) gave negative reactions. The 46 successfully amplified samples were digested individually by *HinfI* and *HaeIII* restriction enzymes. According to the constructed PCR-RFLP algorithms of reference genotypes (Fig. 3), out of the 46 digested samples; 33 (71.8%) (12 human, 14 camel, and 7 pig samples) produced three fragments of 115, 218 and 738 bp when digested by *HinfI*, and two fragments of 235 and 836 bp when digested by *HaeIII* corresponding to G6/7. Two (4.5%) human samples exhibited two fragments of 1035 and 36 bp with *HinfI* and three fragments of 181, 235 and 655 bp with *HaeIII* corresponding to G1. One (2%) pig sample exhibited no digestion by *HinfI* and two fragments of 235 and 836 bp when digested by *HaeIII* corresponding to G5. In 10 samples (21.7%), 2 humans and 8 camels, the digestion patterns did not correspond to a known genotype as inferred from the constructed algorithms of PCR-RFLP of reference genotypes (Table 2, Fig. 4 a-c). It is worth noting that the obtained RFLP pattern banding

Table 1. Banding patterns after digestion of *nad1* gene of *E. granulosus s. l.* reference genotypes with *HinfI* and *HaeIII* restriction enzymes.

Genotype	*HinfI*			*HaeIII*		
	No.[@]	Digestion[#]	Size[*]	No.[@]	Digestion[#]	Size[*]
G1	2	1029	44, 1031	3	236/425	189, 235, 648
G3	3	1029	44, 1031	3	236/425	189, 235, 648
G4	3	727/909	164, 185, 729	Uncut	---	---
G5	Uncut	----	----	2	234	233, 839
G6	3	95/322	97, 230, 751	2	234	233, 839
G7	3	95/322	97, 230, 751	2	234	233, 839
G8	2	323	325, 750	2	235	234, 838
G10	2	95	97, 978	2	234	233, 839
E. felidis	2	322	324, 751	Uncut	---	---

[@]**:** No. of fragments; [#]**:** Digestion position from 5` end; [*]**:** Size of fragments (bp).

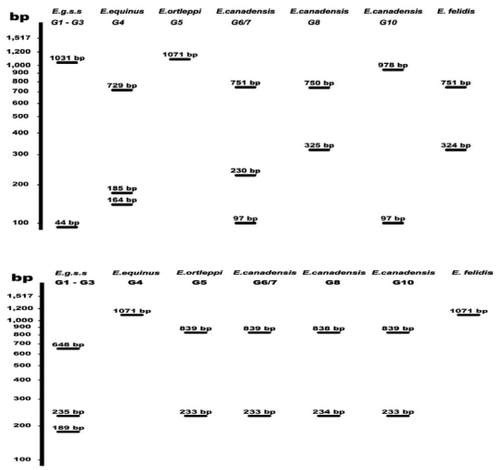

Fig. 1. An algorithm based on *HinfI* digestion of *nad1* gene sequences from *E. granulosus s. l.* reference genotypes (Constructed by Geneious 10.1.3 software).

Fig. 2. An algorithm based on *HaeIII* digestion of *nad1* gene sequences from *E. granulosus s. l.* reference genotypes (Constructed by Geneious 10.1.3 software).

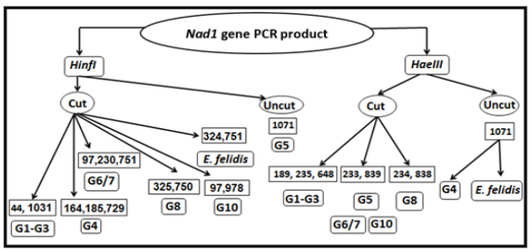

Fig. 3. An algorithm showing expected restriction fragment patterns (bp) and their corresponding genotype based on *HinfI* and *HaeIII* RFLP of *nad1* gene of *E. granulosus s. l.* reference genotypes.

Table 2. Genotypes of human, camel, and pig *E. granulosus* isolates based on PCR-RFLP constructed algorithms of *nad1* gene*

Genotype based on PCR-RFLP algorithms	Isolate	No.	~ %	*HinfI*	*HaeIII*
G6/7	Human Camel Pig	12 14 7	71.8%	115,218,738	235, 836
G1	Human	2	4.5%	36,1035	181,235,655
G5	Pig	1	2.0%	Uncut	235, 836
UD#	Human Camel Camel	2 1 7	21.7%	Uncut Uncut 115,218,738	Uncut Uncut 127,185,759

***:** The obtained RFLP pattern banding sizes were slightly different from the expected PCR-RFLP-constructed algorithm;
#: Undetermined.

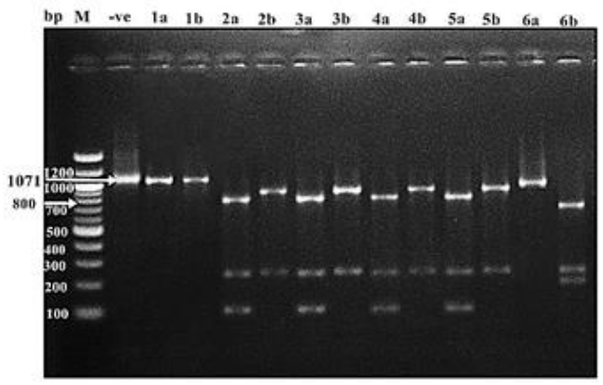

Fig. 4a. An ethidium bromide-stained 2% agarose gel showing RFLP patterns of *E. granulosus nad1* gene from human samples after digestion with *HinfI* (a) and *HaeIII* (b) restriction enzymes. **Lane M:** 100 bp-molecular marker; **lane –ve:** undigested PCR product (1071 bp); **lanes 1a, 1b:** from a human sample with undetermined genotype; **lanes 2a, 2b, 3a, 3b, 4a, 4b, 5a, 5b:** from 4 human samples corresponding to G6/7; **lanes 6a, 6b:** from a human sample corresponding to G1 based on PCR-RFLP algorithms.

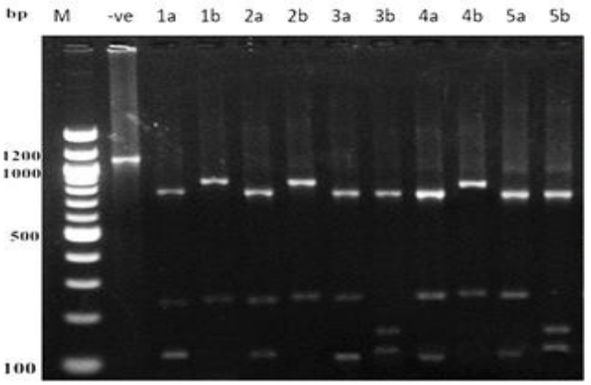

Fig. 4b. An ethidium bromide-stained 2% agarose gel showing RFLP patterns of *E. granulosus nad1* gene from camel samples after digestion with *HinfI* (a) and *HaeIII* (b) restriction enzymes. **Lane M:** 100 bp-molecular marker; **lane –ve:** undigested PCR product (1071 bp); **lanes 1a, 1b, 2a, 2b, 4a, 4b:** from 3 camel samples corresponding to G6/7; **lanes 3a, 3b, 5a, 5b:** from two camel samples with undetermined genotype based on PCR-RFLP algorithms.

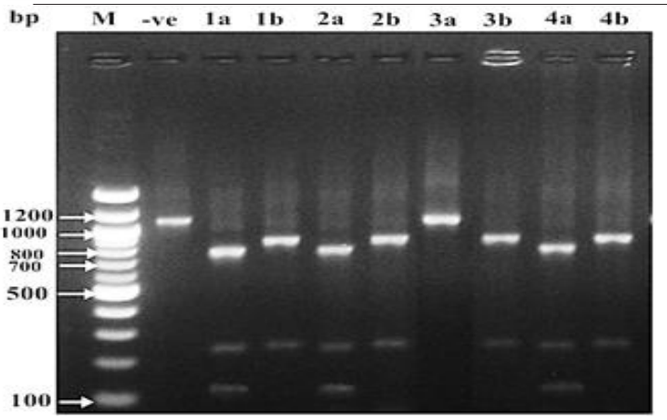

Fig. 4c. An ethidium bromide-stained 2% agarose gel showing RFLP patterns of *E. granulosus nad1* gene from pig samples after digestion with *HinfI* (a) and *HaeIII* (b) restriction enzymes. **Lane M:** 100 bp-molecular marker; **lane –ve:** undigested PCR product (1071 bp); **lanes 1a, 1b, 2a, 2b, 4a, 4b:** from 3 pig samples corresponding to G6/7; **lanes 3a, 3b:** from a pig sample corresponding to G5 based on PCR-RFLP algorithms.

sizes were slightly different from the expected ones as inferred from the constructed algorithm of PCR-RFLP of reference genotypes.

Sequence analysis: To confirm the accuracy of the constructed algorithms of PCR-RFLP of reference genotypes for typing of human and animal isolates and to reveal the genotypes of undetermined specimens; 7 already determined (representing the different restriction patterns) and 4 undetermined samples were selected for sequencing (Table 3). The obtained sequences were subjected to blast search in the NCBI GenBank. Sequence analysis revealed the presence of G6 in 3 human, 5 camel, and one pig samples; G1 in one human sample; G5 in one pig sample. Sequencing results confirmed the genotypes of the already 7 determined ones and revealed the genotypes of the 4 undetermined samples as G6. The genotypes of human, camel, and pig *E. granulosus* samples based on PCR-RFLP algorithms and sequencing of *nad1* gene are shown in table (4).

Table 3. Genotyping by sequencing of 11 isolates representing different and undetermined patterns by PCR-RFLP algorithms of *E. granulosus nad1* gene.

Isolates No.	Genotyping	
	PCR-RFLP algorithms	Sequencing
H1	UD*	G6
H3	G6/7	G6
H9	G1-G3	G1
H10	G6/7	G6
C7	UD*	G6
C10	UD*	G6
C14	UD*	G6
C16	G6/7	G6
C23	G6/7	G6
P3	G5	G5
P7	G6/7	G6

H: Human, **C:** Camel, **P:** Pig, ***UD:** undetermined

Table 4. Genotypes of human, camel, and pig *E. granulosus* samples based on PCR-RFLP algorithms and sequencing of *nad1* gene*

Isolate	No.	Genotype based on PCR- RFLP algorithm and sequencing	%
Human (No. = 16)	14	G6	87.5%
	2	G1	12.5%
Camel (No. = 22)	22	G6	100%
Pig (No. = 8)	7	G6	87.5%
	1	G5	12.5%

***:** Among the 46 samples genotyped; 43 (93.5%), 2 (4.5%), 1 (2%), exhibited G6, G1, and G5 respectively.

DISCUSSION

Genetic characterization of *E. granulosus* populations is crucial for better diagnosis and control of cystic echinococcosis, and for understanding of the taxonomy of the parasite[5,6]. In the present study, RFLP-algorithms were constructed for genotyping of human and animal *E. granulosus* isolates in Egypt based on *in silico* individual digestion of *nad1* gene of reference genotypes G1-G10 and *E. felidis* retrieved from GenBank with two restriction enzymes, *HinfI* and *HaeIII*. The obtained algorithms could differentiate all genotypes except for G1-G3 and G6/G7 when the results were interpreted at the level of both enzymes. At the level of *HinfI* enzyme, RFLP algorithm of the *nad1* gene had greater discriminatory ability in genotyping of *E. granulosus s. l.* than *HaeIII* RFLP algorithm. This implies the value of using more than one restriction enzyme for construction of RFLP interpretation algorithms[12]. Similarly, other investigators developed accurate *in silico* PCR-RFLP targeting *its1* gene[10], *nad1* gene[16], *nad1* and *cox1* genes[12] that discriminated between *E. granulosus s. l.* and most closely related species. However, these assays could not differentiate between

some genotypes as G1-G3 or G6/7 as concluded from our present study. In a recent study by targeting 3 SNPs positions in *nad5* gene, Bonelli *et al*.[22] were able to differentiate between G1 and G3 using TaqMan real time PCR.

PCR-RFLP revealed that 71.8% of samples produced banding patterns corresponding to G6/7, 4.5% exhibited a banding pattern corresponding to G1, and 2% exhibited a banding pattern corresponding to G5. The banding patterns were almost identical to the expected constructed algorithm patterns with minor differences, possibly due to the method of calculation of the band sizes or the dynamics of migration in the gel. In 21.7% of the samples, PCR-RFLP failed to determine the genotypes as deduced from the results of the constructed algorithm probably due to failure or unexpected cutting by either of the two enzymes, or due to technical errors or nucleotide polymorphism. Sequencing of 11 isolates corresponding to different RFLP and undetermined patterns gave matched genotype to those of the 7 determined and revealed the genotypes of the 4 undetermined samples as G6. Based on the results of PCR-RFLP constructed algorithms and sequencing of *E. granulosus nad1* gene, we noticed that G6 is the commonest genotype (93.5%) followed by G1 (4.5%), and G5 (2%). Among human samples, 87.5 % were G6 and 12.5 % were G1. Among camel samples, 100 % were G6. Regarding pig samples, G6 was the commonest genotype in 87.5 %, while G5 was found in 12.5 %. G1 appeared exclusively in humans, and G5 in pigs.

Several studies used PCR-RFLP and/or sequencing techniques targeting mitochondrial *nad1* and *cox1*, and nuclear *its* genes, for genotyping of *E. granulosus* isolates[12,16,23-27]. Accordingly, different genotypes based on the geographic area and intermediate hosts were obtained with predominance of G1 in Tunisia, Uganda, Iran, Kenya[16,24,26-28] and G6 in Sudan[23] with occasional cases of other genotypes. PCR-RFLP could not in all cases distinguish between closely related genotypes as G1, G2, and G3, and G6 and G7 that needed sequencing.

In the present study, it is worth noting that more than one RFLP pattern was obtained for the same genotype especially with *HaeIII* restriction enzyme, which might indicate nucleotide polymorphism and intraspecific strain diversity; although the possibility of failure or unexpected cutting due to technical errors could not be excluded. The polymorphism of the Egyptian G6 isolates could possibly have resulted from the fact that most camels for human consumption in Egypt are imported from Sudan and are the source of *E. canadensis* in Egypt[29]. African isolates particularly those from Sudan and Kenya were found to be highly polymorphic as compared to the European and Middle Eastern isolates[30]. Also, Kinkar *et al*.[8] recorded the genetic diversity of G1 *E. granulosus* global isolates.

The present study using PCR-RFLP and sequencing showed that, except for two cases which were typed as G1 among humans and one case as G5 in pigs, *E. canadenesis* G6 is nearly the commonest genotype among human, camel and pig samples examined. The predominance of G6 in Egypt has been previously documented by several studies[29,31-37]. Alam-Eldin *et al*.[36] reported the finding of G7 in two human isolates and one pig isolate and considered that as the first record of G7 in Egypt. On the other hand, other researchers found G1 common in humans, camels, and sheep in Egypt[38,39].

In conclusion, algorithms based on PCR-RFLP of *nad1* gene are valuable tools for genotyping of *E. granulosus s. l.* Although *HinfI* RFLP algorithm of *nad1* gene had greater discriminatory power than *HaeIII* algorithm for genotyping of the same isolate, both failed to determine the genotype of some isolates. It is important to construct PCR-RFLP algorithms using more than two enzymes and targeting more than one gene to overcome ambiguity of PCR-RFLP patterns. Sequencing is still needed to reveal the genotypes of undetermined or ambiguous isolates. From the epidemiological point of view, the present study revealed that G6 is the commonest genotype in Egypt with few encounters of G1 and G5.

Author contributions: Nassar, DA revised and collected literature, collected the samples, performed PCR-RFLP and genotyping of the parasite. Khalifa, AKE revised and collected literature, helped in sample collection and participated in the practical work of the study. Abou-Seri, HM supervised the practical work, performed the *in silico* RFLP, analyzed the data and shared in writing of the manuscript. Ezz Eldin, HM, and El Wakil, HS designed and supervised the study, revised the results shared in writing and revision of the manuscript. All authors read and approved the final manuscript.

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