Molecular Characterization of Hydrophobic NAD Genes in Echinococcus granulosus Cysts Isolated from Humans and Sheep in Basrah and Thi-Qar Provinces

By Maysoon Khazail Challoob

WORD COUNT 6300
TIME SUBMITTED 07-MAY-2024 05:44PM
PAPER ID 108808657

Word Count – Words: 6300

Molecular Characterization of Hydrophobic NAD Genes in *Echinococcus granulosus* Cysts Isolated from Humans and Sheep in Basrah and Thi-Qar Provinces

Maysoon Khazail Challoob*, Sarmad Awad Mozan AL-Asadi

Department of Biology, College of Education for Pure Sciences, University of Basrah, Basrah, Iraq

Corresponding

Email:
pgs.maysoon.khazail@uobasrah.edu.iq

Word Count – Words: 6300

ABSTRACT

Background: The NADH dehydrogenase family serves as proton (H +) pumps and primary electron acceptors in the respiratory chain. It also represents the main source of oxidative phosphorylation reactions in eukaryotes, which possess three families of NADH dehydrogenases (proton-pumping, non-proton-pumping, and sodium-pumping).

Aims. This research paper aims at identifying and characterizing the NADH genes of these families and getting them investigated in cultured G1 germ cells and hydatid cysts isolated from sheep and humans as well as determining their genotype.

Methods. For the purpose of singling out the families of genes encoded members of peptidase I in *E. granulosus*, a thorough examination was conducted for these genes in the nucleus and mitochondrial genomes of the parasite *E. granulosus*. It included collecting all available sequences of these genes, comparing them with each other, and scrutinizing them in order to determine the identity and number of members of the subunits that make up the first peptidase in the *E. granulosus* parasite. The cells were routinely cultured Hydatids were collected from human (20) and sheep (35) infected livers. Human Hydatids were surgically removed from patients. The contents of the hydatid cysts were separated under sterile conditions. The protoscolices and germinal layers were then kept at -20°C until needed. Sheep livers infected with hydatids were obtained from Central Slaughterhouse. DNA extraction and polymerase chain reaction for 7 hydrophobic dehydrogenase subunit proteins (NADI, NAD2, NAD3, NAD4, NAD5, NAD6, and NAD4L) were done. DNA Sequencing and Bioinformatics Analysis also done.

Results. The results of bioinformatics analysis of the nuclear and mitochondrial genome of the G1 brought to light that it contains (21) subunits of proton-pumping NADH dehydrogenases distributed between the nuclear and mitochondrial genomes. Its number in the mitochondrial genome was 15 (71.43%) subunits and in the nuclear genome 6 subunits (28.57%). The results of the polymerase chain reaction using primers for amplifying the genes of the NADI to NAD4L subunits disclosed their presence at a rate of 100% in both cultured germ cells of the G1 strain and in the primary heads and germinal layer of the cysts isolated from sheep and humans. They had low molecular weights that ranged from 9.9 kDa for the NAD4L subunit to 60.2 kDa for the NAD5 subunit. Moreover, the results unveiled that it possesses regular amino acids in the form of α-helical structures, with similar percentages between 42.86 and 48.28%. The loop structures were in somewhat synonymous proportions, ranging from 51.02 to 57.14. With regard to the strand structures, they were found only in NAD4 and NAD5 in addition to its presence at a rate of 100% in all samples studied. The identity rates were 100% with their counterparts from the G1 (reference), whose hydrophobic subunits were neutralized in the current study based on available genomic data.

Conclusion. Phylogenetic tree analysis displayed that the hydrophobic subunits clustered with their counterparts in the G1, and were far from their counterparts in the G6 and G7, whose hydrophobic subunits were also identified based on the subunits that were identified for the G1 in the current study. It is significant to mention that the hydrophobic subunits are conserved between cultured germ cells of the G1 and between the primary heads and the germ layer of cysts isolated from goats and sheep, as well as between individuals of the G1. In addition, all proton-pumping NADH dehydrogenases can be used in the diagnosis of the very similar G1 and G3.

Keywords: Hydrophobic NAD Genes, *Echinococcus granulosus* , Cysts, parasitic worm, zoonosis

INTRODUCTION

First of all, *Echinococcus granulosus* is a type of parasitic worm with a zoonotic origin, the most harmful in all parts of the world [1]. It lives in the small intestine of dogs and many carnivores as a final host in which it produces eggs, and is excreted into the environment through feces. The infection is transmitted to domestic and wild animals as an intermediate and main host, and is transmitted to humans as an intermediate, Incidental host when eating food and water contaminated with eggs [2]. Humans do not participate in transmitting the disease [3], and upon infection, the larval stage reproduces asexually in the organs (primarily the liver), causing cystic echinococcosis [4], which has a strong pathogenicity and a high mortality rate do to the fact that its growth similar to a malignant tumor [5]. Secondly, the unique feature of the primary cysts within the hydatid cyst is the ability to develop bidirectionally, either into an adult worm in the digestive tract of the dog, or into a secondary cyst in the intermediate host, the human [6]. In Iraq, cystic echinococcosis is hyperendemic and has social and economic impacts targeting humans and their livestock [7]. This is ranked among the most neglected tropical diseases, and also ranks second in the list of food-borne parasitic diseases. A World Health Organization report, made it clear that more than one million people are infected with echinococcosis at any given time [8,9].

Thirdly, the *E. granulosus* genome is the first tapeworm genome to be described. Data analysis exposed that it has complete pathways for glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway [10]. Glycolytic enzymes are the main pathway for energy generation, and are significant molecules for the survival and development of Echinococcus. granulosa [11] because it lacks a digestive, respiratory, and circulatory system [6]. It obtains glucose as an energy source from its host, and metabolizes it through glycolysis and fermentation [12] as well as through the mitochondrial pathways via oxidative phosphorylation. Electrons are transferred from NADH and succinate to oxygen Through the proton-pumping electron transport chain in which ubiquinone (UQ) is used [13].

Furthermore, the molecular sequence of the mitochondrial (mt) genomes, including NADI, NAD2, NAD5, and COXI, has afforded useful genetic information to support compositional and evolutionary analysis and to explore the nature and extent of genetic diversity within Echinococcus [14-16]. Based on the molecular sequence, eight generations of *E. granulosus* have been identified worldwide [17]. It was also demonstrated that G2 is part of G3 and not an independent strain [18], and G9 is part of the G7 strain (16). The G1 is the most wide-ranging in the world [17,19]. As can be seen, some NAD genes were employed in the molecular identification of *E. granulosus* strains. However, the total NAD genes in this parasite have not been determined and characterized. Thus, this study aimed to determine and characterize the NAD genes in *E. granulosus* and to investigate these genes in cultured germinal cells (the G1 strain), sheep, and human hydatid cysts [19].

METHOD

Bioinformatics Analysis

For the purpose of singling out the families of genes encoded members of peptidase I in *E. granulosus*, a thorough examination was conducted for these genes in the nucleus and mitochondrial genomes of the parasite *E. granulosus* (sheep G1. Such examination was carried out using bioinformation analyzes available in the databases of the National Center for Biotechnology Information (NCBI). It included collecting all available sequences of these genes, comparing them with each other, and scrutinizing them in order to determine the identity and number of members of the subunits that make up the first peptidase in the *E. granulosus* parasite.

Echinococcus Granulosus Cell Culture

E. granulosus strain G1 cells were obtained as a gift from Dr. Sarmad AL-Asadi and Dr. Ali AL-Ali. The cells were routinely cultured in 25-cm^2 cell culture flasks (Corning) at a temperature of 37 ^{o}C in an atmosphere of 5% (v/v) CO2 in air in a medium consisting of RPMI 1640 culture medium supplemented with 10% (v/v) mfoetal bovine serum (FBS, Life Technologies), 100 units ml penicillin/streptomycin and 4 mM L-glutamine. After trypsinization using trypsin-EDTA, the number of viable cells was determined by staining the cells with Trypan blue dye and then counting them using a hemocytometer. The cells were subcultured every three days.

Hydatid Collection

Hydatids were collected from human (20) and sheep (35) infected livers during the period from November 2022 to January 2023. Human Hydatids were surgically removed from patients at Al-Fayhaa Hospital in Basrah province. The contents of the hydatid cysts were separated under sterile conditions. The protoscolices and germinal layers were then kept at -20°C until needed. Sheep livers infected with hydatids were obtained from Central Slaughterhouse in Basrah province and in Thi-Qar (Fig. 1)

Figure 1. Hydatids are collected from sheep and humans**. A** represents the protoscolices of *E. granulosus* and **B** represents the germinal layer**. C** represents human hydatids.

DNA EXTRACTION

It is significant to pinpoint that all deoxyribonucleic acid (DNA) was extracted from cultured germ cells of the G1 sheep strain and from the primary heads and germinal layer isolated from human and female hydatid cysts using a DNA extraction kit prepared by Geneaid. It was operated through following the manufacturer's steps for isolating DNA from tissues according to the leaflet attached to the kit. The concentration of DNA extracted from samples was estimated using a Quantus TM fluorometer (Promega, USA). gDNA was maintained at −20°C until needed.

Polymerase Chain Reaction

First, this technology was utilized to detect the presence and specification of (7) hydrophobic dehydrogenase subunit proteins (NADI, NAD2, NAD3, NAD4, NAD5, NAD6, and NAD4L) in both DNA samples extracted from cultured germ cells of the G1 strain of sheep and DNA samples extracted from the primary heads and germinal layer isolated from humans and sheep. For adequate results, specialized primers were designed to target the proteins of the hydrophobic subunits (NADI, NAD2, NAD3, NAD4, NAD5, NAD6, and NAD4L) individually manipulating the Primer3Plus program (Table 1).

Second, these primers successfully enlarged on the seven subunit genes (NADI to NAD4L) with lengths of 676, 633, 246, 780, 899, 336, and 197 base pairs, respectively. Each PCR reaction was of 25 µl of Promega master mix, 7 µl of gDNA, and 14 µl of nuclease-free water. At 95 °C one cycle was executed for five minutes, 40 cycles were performed at 95 °C for 30 seconds, at 55 °C or 56°C (with NAD5) for 30 seconds, at 72 °C for 30 seconds, with an extension step. Final ten minutes at 72 °C. A 1.5% (wt/vol) agarose gel was employed to separate PCR production. These products have been cleaned using the *Promega* cleaning system.

Table 1 Primers designed and used in the current study

Gene		5` ---------- 3`	bp
NADI	F	TTTGTTGCAGAGGTTTGCTG	676
	R	ACGAACACGTGGTAATGTCG	
NAD2	F	GGTTGGCAATTGTTCCTTCA	633
	R	TGCCCACACACACACTTATCT	
NAD3	F	GTTTGAGTGGGGCAGTTCTT	246
	R	TAAACCCAACACATACCC	
NAD4	F	TCCGTATTCTGAGCGGTTTT	780
	R	AATGGAACCAATCCACCAAA	
NAD5	F	GTCATTATTTCGGCGGTGAT	994

	R	ACCACCAATCCAGAACCAAA	
NAD6	F	TGTTGGTGGTGAATGCGTTA	336
	R	CCCTCAAAACCAAAAATCCA	
NAD4L	F	TTTTTAACTGTTGGGCGTTTTT	197
	R	TCCAAACAAGAAGAACACTCTCA	

DNA Sequencing and Bioinformatics Analysis

25 µL of purified PCR product of the study samples (2 from human and 1 cultured germ cells of G1 strain from sheep), and (10) µL each of forward and reverse primers specifically designed for each of the (7) hydrophobic subunits were sent to Macrogen Company in South Korea for sequencing. The DNA of each sample was analyzed using the Genetic Analyzer device, then the sequence alignment and phylogenetic tree were performed using the *Mega X* program.

Statistical Analysis

Statistical analysis was accomplished using SPSS (Statistical Package for Social Sciences) to analyze the data in the current study, and the Chi-square test was applied.

RESULTS

Bioinformatics Analysis

The results of the bioinformatics analysis of the mitochondrial genome (mt DNA) and the nuclear DNA of the sheep strain (G1) disclosed important genetic information, including its possession of (21) subunits of peptidase I, and that most of these subunits are present in the mitochondrial genome, as their number reached (15) subunits (71.43%), with the presence of 6 subunits (28.57). %) in the nucleus genome. Besides, it was made clear that all of these subunits belong to the family of proton-pumping dehydrogenase enzymes, and the Echinococcus granulosus parasite does not contain the family of non-proton-pumping dehydrogenases or sodium-pumping enzymes. The results of the statistical analysis also brought to light that there were significant differences between them (Figure 2).

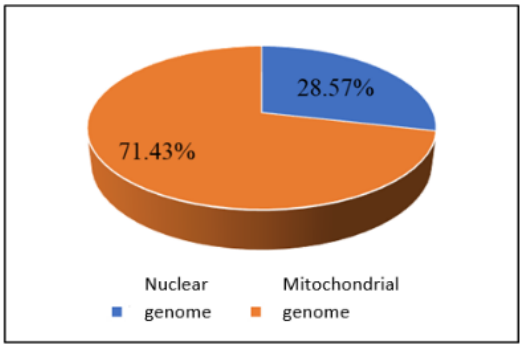

Figure 2. Percentage of subunit genes encoded to proton-pumping dehydrogenase enzymes in the nucleus genomics and mitochondria of *E. granulosus*.

The Molecular Weights of NAD Hydrophobic Proteins

The results of the mass study of hydrophobic dehydrogenase proteins exhibited that members of this group had low molecular weights that ranged from 9.9 to 60.2 kilodaltons (Table 2). The highest molecular weight was (60.2 kDa) for the NAD5 subunit protein, and the lowest molecular weight was (9.9 kDa) for the NAD4L subunit protein.

Table 2. Molecular Weights of Hydrophobic Subunit Proteins

Type	Amino acids	Molecular weight(kDa)
NADI	297	33.5
NAD2	293	34.3
NAD3	115	13.6
NAD4	419	47.7
NAD5	532	60.2
NAD6	151	17.4
NAD4L	86	9.9

The Structural Model of NAD Hydrophobic Proteins

The results of the structural study of hydrophobic dehydrogenase proteins flashed that all members of these subunits bear amino acids that are regular in the form of α-helix structures and others regular in the form of loops or turns. However, these structures vary in number from one unit to another (Figure 3). The highest number of helical (22) and toroidal (25) structures was in NAD5, and the lowest number of helical (3) and toroidal (4) structures was in NAD4L (Table 3). The results also showed that most of the subunits (with the exception of the NAD4 and NAD5 subunits) lack amino acids organized in the form of β-strand sheet. The contrary is that the NAD4 and NAD5 subunits each have a pair of β sheet.

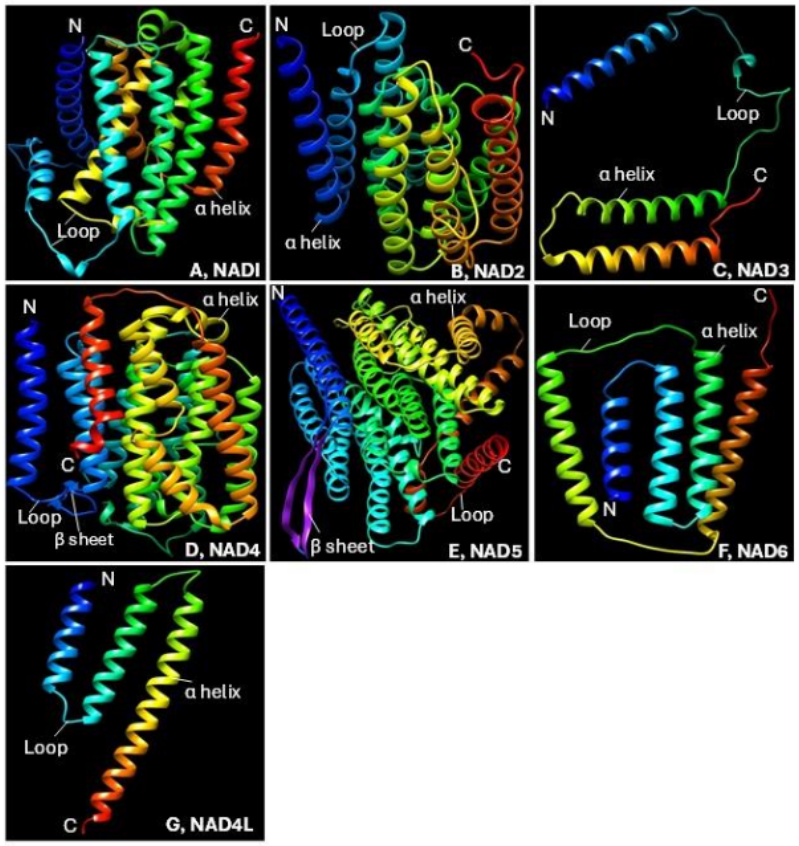

Figure 3. Structural models of hydrophobic NAD proteins. The letters A to G represent NADI to NAD4L.

Table 3. Structural components of hydrophobic subunit proteins.

Type	α helix	β Strand	loop
NADI	14	-	15
NAD2	13	-	14
NAD3	4	-	5
NAD4	18	2	21
NAD5	22	2	25
NAD6	5	-	6
NAD4L	3	-	4

Besides, the results of the study declared the structure of the proteins of the hydrophobic units despite the difference in the number of amino acids for each subunit, the percentage of helical structures was similar in all subunits, ranging from 42.86 to 48.28%. Moreover, the cyclic structures exhibited somewhat similar percentages among the seven hydrophobic units, as the percentage ranged from 51.02 to 57.14.

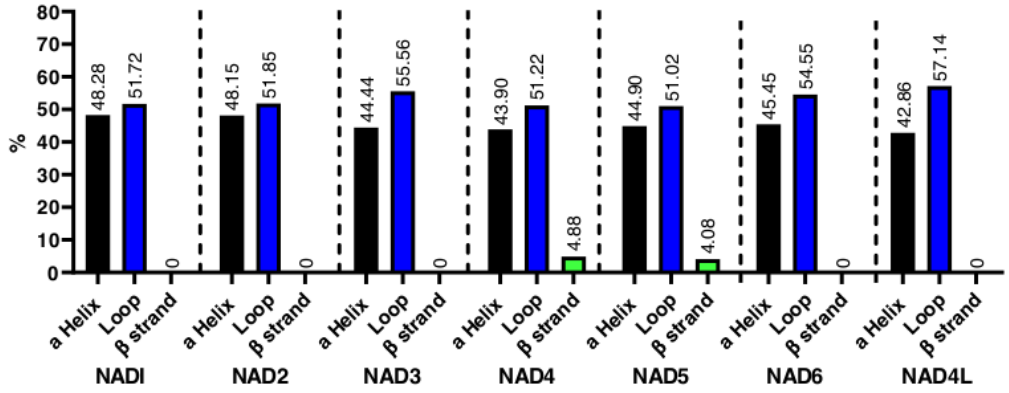

Figure 4. Percentage of α helix and strip structures and loops structures or turns in structural models of hydrophobic units.

Detection of hydrophobic NAD Genes

The seven hydrophobic subunits of NADH genes were successfully amplified using PCR in the germinal cells of the G1 strain and germinal layers and protoscolices of hydatids isolated from human and sheep livers (Figure 5). In addition to this, our findings showed that these subunits, NADI to NAD4l, were present in geminal cells and all protoscolices of examined samples (Table 4).

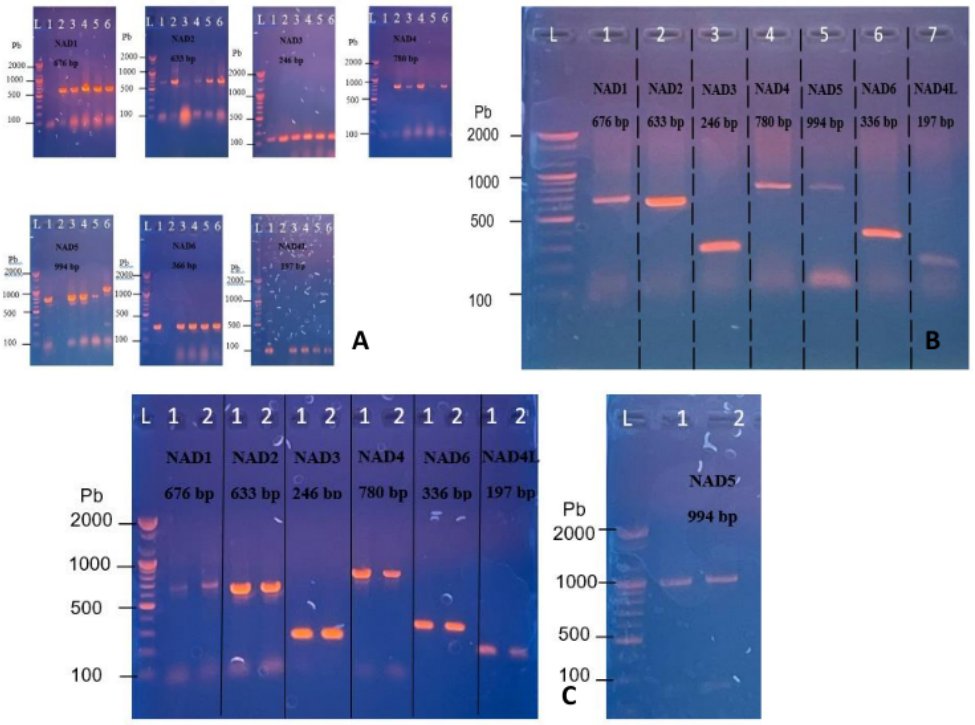

Figure 5. Electrophoresis of PCR products of the seven hydrophobic subunits. A. in the cell line of the G1 strain sheep. B. in hydatids isolated from sheep. C. in hydatids isolated from human

Table 4. The hydrophobic NAD genes in cultured cells and in human and sheep hydatids.

Subunit	Cell line	Sheep (35 hydatids)		Human (20 hydatids)	
		Protoscolices	Germinal layer	Protoscolices	Germinal layer
NADI	+	+	+	+	+
NAD2	+	+	+	+	+
NAD3	+	+	+	+	+
NAD4	+	+	+	+	+
NAD5	+	+	+	+	+
NAD6	+	+	+	+	+
NAD4L	+	+	+	+	+

+represents the presence of the subunit

DNA Sequencing analysis

Purified PCR products of protoscolices and germinal layers of human hydatids and cultured cells (Sheep strain) were sequenced in both directions to each hydrophobic NAD gene using gene-specific primers. In this field of study, the cultured cells of the G1 strain were also used as a representative of the sequence of hydrophobic subunits in sheep hydatids. After merging and editing DNA sequencing of each subunit, the sequence was examined against Blast search. The NAD sequences of cultured cells were deposited in GenBank via the NCBI website. As can be seen, all human hydatid sequences (germinal layers and protoscolices) had 100% identity with each other's. Thus, for each NAD subunit of human hydatids, the consensus sequence of germinal layers and protoscolices was also deposited in GenBank. The accession numbers of the current study samples are mentioned in (Table 5).

Table 5. Accession numbers of the present study samples.

Subunit	Cultured cells (Sheep strain)	Human hydatids	
		Germinal layer	protoscolices
NADI	PP116552.1	PP391477.1	PP391476.1
NAD2	PP116553.1	PP391479.1	PP391478.1
NAD3	PP116554.1	PP391481.1	PP391480.1
NAD4	PP116555.1	PP391483.1	PP391482.1
NAD5	PP116556.1	PP391485.1	PP391484.1
NAD6	PP116557.1	PP391487.1	PP391486.1
NAD4L	PP116558.1	PP391489.1	PP391488.1

Phylogenetic Analysis

The evolutionary tree was built based on the amino acid sequence of the seven hydrophobic subunit proteins of complex I of the current study samples and their counterparts in the G1, G6, and G7 strains (Fig. 6). The analysis results of cultured cells (sheep strain) revealed that NADI to NAD4L sequences were grouped with their counterparts in the NCBI reference G1 strain (Australia) and were separated from other G6 and G7 strain (Fig.6A). Similarly, the phylogenetic

analysis of human hydatid sequences (NADI to NAD4L) of either germinal layers or protoscolices were also clustered with their counterparts in the NCBI reference G1 strain (Australia) and were separated from other G6 and G7 strain (Fig.6B).

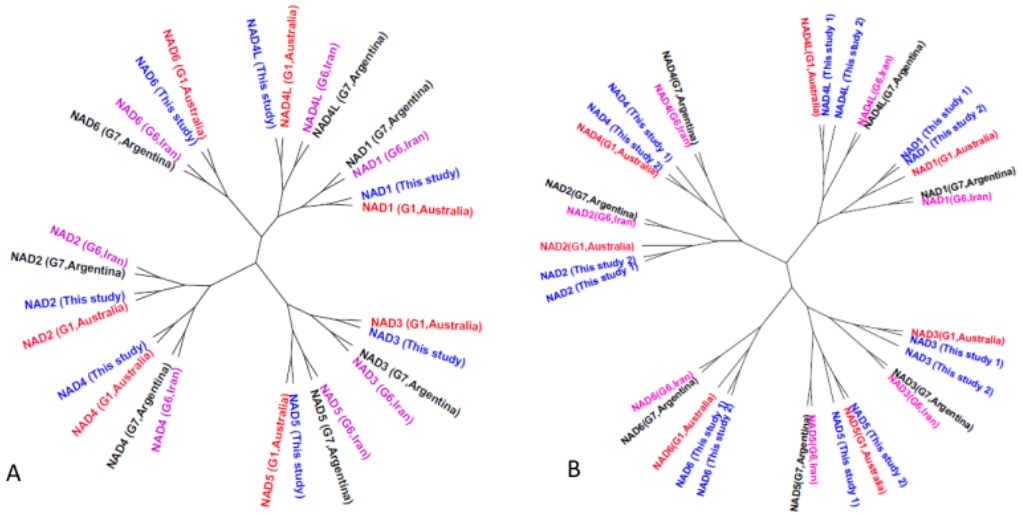

Figure 6. The evolutionary tree of the current study samples was built based on the amino acids of hydrophobic subunits in the present study samples and G1 (Australia), G6 (Iran), and G7 (Argentina) strains that are chosen based on their available genome data. The A letter represents cultured cells (sheep strain) while the B letter represents human hydatids.

In human hydatids, this study 1 is the consensus sequence from germinal layers while this study 2 is the consensus sequence from protoscolices). NCBI accession numbers are in (Table 6).

Table 6. NCBI accession numbers of hydrophobic NAD proteins used in the phylogenetic tree.

Subunit	NCBI accession numbers		
	G1 strain (Australia)	G6 strain (Iran)	G7 strain (Argentina)
NADI	YP_009688057.1	YP_009505042.1	YP_009505054.1
NAD2	YP_009688056.1	YP_009505041.1	YP_009505053.1
NAD3	YP_009688058.1	YP_009505043.1	YP_009505055.1
NAD4	YP_009688054.1	YP_009505039.1	YP_009505051.1
NAD5	YP_009688050.1	YP_009505035.1	YP_009505047.1
NAD6	YP_00968849.1	YP_009505046.1	YP_009505058.1

NAD4L	YP_009688053.1	YP_009505038.1	YP_009505050.1

Percent Identity

The percentage of identity between the hydrophobic NAD proteins of the current study samples and their counterparts the reference G1strain (Australia) was shown in (Table 7). The NADI to NAD4L amino acids sequences of cultured germinal cells and human hydatids had 100% identity within each other and with their counterparts in the reference G1 strain (Australia). Moreover, no differences were noted in these proteins when their amino acids were lined up.

Table 7. The percent identity of hydrophobic NAD proteins of the percent samples with NADs of NCBI G1 strain.

Subunit	NCBI Reference G1strain (Australia)	Sheep strain	Human hydatids	
		Cultured cells (%)	Germinal layer (%)	Protoscolices (%)
NADI	YP-009688057.1	100	100	100
NAD2	YP-009688056.1	100	100	100
NAD3	YP-009688058.1	100	100	100
NAD4	YP-009688054.1	100	100	100
NAD5	YP-009688050.1	100	100	100
NAD6	YP-00968849.1	100	100	100
NAD4L	YP-009688053.1	100	100	100

DISCUSSION

First of all, it is of high significant to refer to the fact that the growth of the Echinococcus granulosus parasite and its ability to continue to survive is reliant mainly on biological systems for generating energy, as in other eukaryotic organisms, which use glucose as the main source of energy and break it down through glycolysis, fermentation, or through mitochondrial pathways associated with oxidative phosphorylation through the electron transport chain [12]. Studying the mt DNA genes of *E. granulosus* is essential since it is the source of useful genetic information to discover the nature and extent of genetic diversity within *Echinococcus granulosus*, distinguishing between very similar strains. Besides, it is so important because it works on

identifying the most common strain, as well as understanding the causes of disease transmission and development novice health treatments and vaccines to combat echinococcosis.

Second, the NADH of mitochondria (Complex I) Complex I (NADH ubiquinone oxidoreductase) is one of the five largest known membrane protein complexes (complexes I, II, III, and IV, V). It has a central role in energy generation [20]. It stimulates the first step of the electron transfer chain in mitochondria [21], and behaves as a membrane-bound proton pump that pushes four protons across the membrane using the energy resulting from redox reactions [22] and is a significant source for the production of reactive oxygen species (ROS) [23].

Besides, there are three families of proton-pumping non-proton-pumping, and sodium-pumping NADH dehydrogenase enzymes [24]. There are no local or global studies on these families in the Echinococcus granulosus parasite. Therefore, the current paper aims at characterizing and verifying these families in the G1 strain of the Echinococcus granulosus parasite as well as depicting the genotype of hydrophobic dehydrogenase enzymes.

Furthermore, the results of the present study pinpointed that the genome of the Echinococcus granulosus parasite contains 21 subunits. While previous studies stated that the number of subunits in trypanosomatids reaches more than 60 subunits [25], while in Escherichia coli bacteria, the number reaches 13 subunits [26]. The results also brought to light that all of these units belong to the family of proton-pumping NADH dehydrogenase enzymes. This is consistent with the previous study that evidenced the existence of the proton-pumping NADH dehydrogenase family in real bacteria and the vast majority of eukaryotic organisms with the exception of *S.cerevisica* [27]. Results confirmed their lack of the unpleasant hydrogen disputers family that was found in the true bacteria, the elderly sortie, yeasts, innate, and plants [28,29]. It also lacks the family of sodium-pumping dehydrogenase enzymes that have been found in many bacteria, including many marine species and in some known types of human pathogens [30,31]. The results also strengthened the fact that all subunits are distributed between the nuclear and mitochondrial genomes, as the nuclear genome has 6 subunits (28.57%), while the mitochondrial genome has 15 subunits (71.43%).

Likewise, these results are consistent with previous studies that disclosed that the first bacterial complex, NAD-1, possesses 14 subunits in the plasma membrane [32] and the presence of all of these subunits in the first complex of eukaryotic organisms [33]. There are more than 30 additional subunits of complex I in eukaryotes, which led to an increase in its molecular mass from 550 KDa to 1000 KDa [34]. The numerous additional subunits come out to be a special feature of mitochondrial complex I, and are all nuclear encoded. It has been speculated that these subunits form a scaffold around the 14 subunits, and prevent high-energy electrons from escaping from the complex, and thus hinder the formation of reactive oxygen species [35]. The exact roles of most of these subunits are unknown, but some of them have a role in stabilizing

the complex and regulating its activity and assembly [34]. In this study, it is practicable that the six additional subunits of the parasite Echinococcus granulosus have a role in assembling and stabilizing the first complex too.

Also, proteins are large, complex molecules (polymers) consisting of monomer structural units consisting of an amine group (NH2-) representing one end of the protein and a carboxyl group (COOH- representing the other end). They are organized in the form of a long chain of amino acids. Each protein has four distinct levels of protein structure, which are primary, and secondary, tertiary and fourth. Polypeptide chains are folded and bent in the form of helical structures, which are the most common, and in the form of stripe structures, loops structures, or turns, which are the least common. Th[36] structures give the protein stability and give it a functional three-dimensional shape [36]. This is consistent with the results we reached from the structural study of hydrophobic dehydrogenase proteins. All members of these subunits contain amino acids that are organized in the form of α-helical structures and others regular in the form of loops or turns. These structures were more numerous in the NAD4 subunit (18 helical structures), and 21 loops structures (and in the NAD5 subunit, there are 22 helical structures and 25 loops structures). As for the stripe structures, they were detected only in the subunit, NAD4 and NAD5, with an average of two pairs of stripe structures in each subunit.
Additionally, the results of the mass of hydrophobic proteins (NADI, NAD2, NAD3, NAD4, NAD5, NAD6, and NAD4L) analysis highlighted that the total molecular weights reached (216.6 kDa), which are (33.5, 34.3, 13.6, 47.7, 60.2, 17.4, and 9.9) kDa, respectively, which range from the highest molecular weight (60.2 kDa) for the NAD5 subunit protein and the lowest molecular weight (9.9 kDa) for the NAD4L subunit protein. These results are harmonious with the results of previous studies in that they are close in molecular weight and that the NAD5 subunit has the highest molecular weight and the NAD4L subunit has the lowest molecular weight (including a previous study of the proteins of bovine mitochondrial complex I (NADI, NAD2, NAD3, NAD4, NAD5, and NAD4L) except for NAD6).

It revealed that the molecular weights are (35.6, 39.2, 13.0, 52.0, 68.2, and 10.7) kDa, respectively, and the total is (218.7 kDa), and that the highest molecular weight (68.2 kDa) is for the NAD5 subunit protein, and the lowest molecular weight is (10.7 kDa).) of the NAD4L subunit protein (25).It also was in harmony with a previous study that depicted that the molecular weights of the proteins of complex I of T. thermophilus bacteria (NADI, NAD2, NAD3, NAD4, NAD5, NAD6, and NAD4L) are (41.0, 44.9, 13.1, 49.4, 65.2, 18.4, and 10.0) and the total is (242.5 kDa). The highest molecular weight (65.2 kDa) is for the NAD5 subunit protein and the lowest molecular weight (10.0 kDa) is for the NAD4L subunit protein [37].

The results also displayed a difference in the number of amino acids for the hydrophobic subunit proteins, ranging from the largest number (538 amino acids) is for the NAD5 subunit protein and the smallest number (68 amino acids) is for the NAD4L subunit protein. Also, the results of the study unveiled the structure of the proteins of the hydrophobic units despite the difference in the numbers of amino acids for each subunit, the percentage of helical structures was similar in all subunits, ranging from 42.86 to 48.28%. The cyclic structures demonstrated similar percentages, as the percentage ranged from 51.02 to 57.14%, while for the ribbon structures, the percentage ranged between 4.88% in the NAD4 subunit and 4.08% in the NAD5 subunit, and the percentage was 0% in the other subunits because they lack stripe structures.

It is significant to disclose that such convergence in the percentages of these structures in all hydrophobic subunits gives stability to the proteins of the hydrophobic units, and the NAD4 and NAD5 subunits are more stable than the other subunits. It is so because they possess three types of helical, turns, and loops structures, and also possess the largest number of amino acids, and thus have molecular high weight. It is possible that the stability of the protein enlarges with increasing molecular weight. The subunits are divided into 7 hydrophilic subunits within the periplasmic arm, encoded by the nuclear genome, and 7 hydrophobic subunits within the transmembrane arm, which in most eukaryotes (cattle, *Arabidopsis thaliana*, and trypanosomes) are encoded by mitochondrial genome [33, 38-40]. Similarly, in *E. granulosus*, the hydrophobic dehydrogenases were present in the mitochondrial genome of geminal cells, protoscolices, and germinal layers.

This study was identified by Bioinformatics and PCR, the seven NAD hydrophobic genes. While in a previous study, AL-Asadi *et al*. (2022) fully made a description of the NAD1 subunit proteins only from the hydrophobic subunits of samples isolated from human liver. Most previous studies have focused on the NADI, NAD2, or NAD5 subunits in distinguishing between *E. granulosus*. strains. In the future, it is likely that these other sequences of the 7 hydrophobic subunit proteins (NAD3, NAD4, NAD6, and NAD4L) will be used to distinguish at the molecular level between the very similar G1 and G3 strains [19].

The results of this study also brought to light that the interpretation of the amino acids of the subunit proteins of both the cell line and samples isolated from humans revealed that they are completely and 100% identical when compared with their counterparts from the G1 (reference) sheep strain, whose hydrophobic subunits were neutralized in this study based on genome data. Available. The genotypes of *E. granulosus sensu* lato are of great variation in their host selectivity and pathogenicity [41].

It was recently comprehended that the hydatid cyst parasite dominates eight strains (G1-G10) divided on the molecular basis of NAD1 genes [19]. The genotypes were determined as G1 (sheep strain), G6 (camel strain), G5 (cattle strain), G4 (horse strain), G3 (buffalo strain), G2

(Tasmanian) G7 (pig strain) G9 (variant pig strain), G8 (American cervid strain) and G10 (Fennoscandian cervid strain) [42].

It is viable that these 7 hydrophobic subunits of complex I of the Echinococcus granulosus parasite are similar in number to their counterparts in these strains but may differ in their amino acid arrangement. Therefore, the results of the analysis of the evolutionary tree of the subunits of the samples of the current study, which were built on the basis of amino acids, when compared with their counterparts of the subunits in the G1, G6, and G7 strains, demonstrated that all the samples belong to the G1 strain. Besides, it was found that the G6 and G7 strains were identified with their hydrophobic subunits, also based on the subunits identified for the G1 strain in the current study. In Basrah and Thi-Qar, G1 is the most common strain that infects human, sheep, and cattle (AL-Asadi et al., 2021; Al-Asadi et al., 2022). This was consistent with our findings based on all hydrophobic subunits (NAD1-NAD4L). The G1 strain is responsible for most human cases of echinococcosis worldwide and is the familiar type in the Middle East [43,44].

CONCLUSIONS

Echinococcus granulosus has 21 proton pump subunits. These subunits are from a Proton pump NADH dehydrogenase family. The hydrophobic parts (NADI to NAD4L) were detected in the protoscolices and germinal layers of hydatids. NADI to NAD4L were also present in cultured geminal cells. These subunits were shared 100% identity with their counterparts in the G1 sheep strain (identified from genomes in this study). Most of the structural complexes of these hydrophobic subunits do not contain strand structures. Proton pump NADH dehydrogenase can be employed to diagnose the very similar G1 and G3 strains. Totally, this study introduces for the first time more details about NAD subunits in hydatids isolated from different intermediated hosts.

Disclosure

None

REFERENCES

1-Shao, G., Hua, R., Song, H., Chen, Y., Zhu, X., Hou, W., ... & Yang, G. (2023). Protective efficacy of six recombinant proteins as vaccine candidates against Echinococcus granulosus in dogs. *PLOS Neglected*

Tropical Diseases, *17*(10), e0011709.

2-AlSalman, A., Mathewson, A., Martin, I. W., Mahatanan, R., & Talbot, E. A. (2023). Cystic Echinococcosis in Northern New Hampshire, USA. *Emerging Infectious Diseases*, *29*(5), 1057.

3-Golemanov, B., Grigorov, N., Mitova, R., Genov, J., Vuchev, D., Tamarozzi, F., & Brunetti, E. (2011). Efficacy and safety of PAIR for cystic echinococcosis: experience on a large series of patients from Bulgaria. *The American journal of tropical medicine and hygiene*, *84*(1), 48.

4-Craig, P. S., McManus, D. P., Lightowlers, M. W., Chabalgoity, J. A., Garcia, H. H., Gavidia, C. M., ... & Schantz, P. M. (2007). Prevention and control of cystic echinococcosis. *The Lancet infectious diseases*, *7*(6), 385-394.

5-Ren, B., Chen, X., Lei, P., Hou, L., Wang, H., Zhou, Y., ... & Yuan, J. (2021). The relationship between preoperative systemic immune inflammation index and prognostic nutritional index and the prognosis of patients with alveolar hydatid disease. *Frontiers in Immunology*, *12*, 691364.

6-Smyth, J. D. (1990). *In vitro cultivation of parasitic helminths*. CRC press.

7-Benyan, A. K. Z., Mahdi, N. K., Abdul-Amir, F., & Ubaid, O. (2013). Second reported case of multilocular hydatid disease in Iraq. *Qatar Medical Journal*, *2013*(1), 5.

8-Food and Agriculture Organization of the United Nations/World Health Organization. Multicriteria-based ranking for risk management of food-borne parasites. Microbiological Risk Assessment Series No. 23. Rome: FAO/WHO; 2014.

9-World Health Organization. (2012). *Accelerating work to overcome the global impact of neglected tropical diseases: a roadmap for implementation* (No. WHO/HTM/NTD/2012.5). World Health Organization.

10-Zheng, H., Zhang, W., Zhang, L., Zhang, Z., Li, J., Lu, G., ... & Wang, S. (2013). The genome of the hydatid tapeworm Echinococcus granulosus. *Nature genetics*, *45*(10), 1168-1175.

11-Xin, Q., Yuan, M., Lv, W., Li, H., Song, X., Lu, J., & Jing, T. (2021). Molecular characterization and serodiagnostic potential of Echinococcus granulosus hexokinase. *Parasites & Vectors*, *14*, 1-9.

12-Ritler, D., Rufener, R., Li, J. V., Kämpfer, U., Müller, J., Bühr, C., ... & Lundström-Stadelmann, B. (2019). In vitro metabolomic footprint of the Echinococcus multilocularis metacestode. *Scientific reports*, *9*(1), 19438.

13-Marr, J., & Muller, M. (Eds.). (1995). *Biochemistry and molecular biology of parasites*. Elsevier.

14-Manterola, C., Totomoch-Serra, A., Rojas, C., Riffo-Campos, Á. L., & García-Méndez, N. (2021). Echinococcus granulosus sensu lato genotypes in different hosts worldwide: a systematic review. *Acta parasitologica*, 1-25.

15-Bhutani N, Kajal P. Hepatic echinococcosis: A review. Ann Med Surg. 2018 Dec 1;36:99-105.

16-Laurimäe T, Kinkar L, Romig T, Umhang G, Casulli A, Omer RA, et al. Analysis of nad2 and nad5 enables reliable identification of genotypes G6 and G7 within the species complex Echinococcus granulosus sensu lato. Infect Genet Evol. 2019; 74: 1-9. https://doi.org/10.1016/j.meegid.2019.103941 12.

17-AL-Asadi SAM, Hansh WJ, Awad A-HH. Employing NADH Dehydrogenase Subunit 1 in the Determination of Echinococcus granulosus Strain in Sheep, Cattle and Human in Thi-Qar Province, Iraq. Baghdad Sci J. 2021; 18(2): 238-46. http://dx.doi.org/10.21123/bsj.2021.18.2.0238

18-Kinkar L, Laurimäe T, Sharbatkhori M, Mirhendi H, Kia EB, Ponce-Gordo F, et al. New mitogenome and nuclear evidence on the phylogeny and taxonomy of the highly zoonotic tapeworm Echinococcus granulosus sensu stricto. Infect Genet Evol. 2017; 52: 52-8. https://doi.org/10.1016/j.meegid.2017.04.023

19-AL-Asadi, S. A. M., & Awad, A. H. H. Complete characterization of NADH dehydrogenase subunit 1 gene in human hydatid cysts. Baghdad Sci J. 2022; https://doi.org/10.21123/bsj.2023.8094

20-Hirst, J. (2013). Mitochondrial complex I. *Annual review of biochemistry*, *82*, 551-575.

21-Schultz, B. E., & Chan, S. I. (2001). Structures and proton-pumping strategies of mitochondrial respiratory enzymes. *Annual review of biophysics and biomolecular structure*, *30*(1), 23-65.

22-Jones AJY, Blaza JN, Varghese F,Hirst J. 2017. Respiratory complex I in *Bos taurus* and *Paracoccus denitrificans* pumps four protons across the membrane for every NADH oxidized. *J. Biol. Chem.* 292:4987–95

23-Chouchani, E. T., Methner, C., Nadtochiy, S. M., Logan, A., Pell, V. R., Ding, S., ... & Murphy, M. P. (2013). Cardioprotection by S-nitrosation of a cysteine switch on mitochondrial complex I. *Nature medicine*, *19*(6), 753-759.

24-Kerscher, S., Dröse, S., Zickermann, V., & Brandt, U. (2008). The three families of respiratory NADH dehydrogenases. *Bioenergetics: Energy Conservation and Conversion*, 185-222.

25-Carroll, J., Fearnley, I. M., Skehel, J. M., Shannon, R. J., Hirst, J., & Walker, J. E. Bovine complex I is a complex of 45 different subunits. JBC, 2006. 281(43), 32724-32727. https://doi.org/10.1074/jbc.M607135200

26-Yagi, T., Yano, T., Di Bernardo, S., & Matsuno-Yagi, A. (1998). Procaryotic complex I (NDH-1), an overview. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1364*(2), 125-133.

27-Melo, A. M., Bandeiras, T. M., & Teixeira, M. New insights into type II NAD (P) H: quinone oxidoreductases. J. Mol. Microbiol., 2004. *68*(4), 603-616. https://doi.org/10.1128/mmbr.68.4.603-616.

28-Kerscher, S. J. (2000). Diversity and origin of alternative NADH: ubiquinone oxidoreductases. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1459*(2-3), 274-283.

29-Melo, A. M., Duarte, M., Møller, I. M., Prokisch, H., Dolan, P. L., Pinto, L., ... & Videira, A. (2001). The external calcium-dependent NADPH dehydrogenase from Neurospora crassa mitochondria. *Journal of Biological Chemistry*, *276*(6), 3947-3951.

30-Kogure, K. (1998). Bioenergetics of marine bacteria. *Current Opinion in Biotechnology*, *9*(3), 278-282.

31- Häse CC, Fedorova ND, Galperin MY, Dibrov PA (2001) Sodium ion cycle in bacterial pathogens: evidence from cross-genome comparisons. Microbiol Mol Biol Rev 65:353–370

32-Yagi, T., & Matsuno-Yagi, A. (2003). The proton-translocating NADH− Quinone oxidoreductase in the respiratory Chain: the secret unlocked. *Biochemistry*, *42*(8), 2266-2274.

33-Rasmussen T, Scheide D, Brors B, Kintscher L, Weiss H, Friedrich T Identification of two tetranuclear FeS clusters on the ferredoxin-type subunit of NADH: ubiquinone oxidoreductase (complex I). Biochem. 2001. 40(20):6124–6131. https://doi.org/10.1021/bi0026977

34-Padavannil, A., Ayala-Hernandez, M. G., Castellanos-Silva, E. A., & Letts, J. A. (2022). The mysterious multitude: structural perspective on the accessory subunits of respiratory complex I. *Frontiers in Molecular Biosciences*, *8*, 798353.

35-Guénebaut, V., Schlitt, A., Weiss, H., Leonard, K., & Friedrich, T. (1998). Consistent structure between bacterial and mitochondrial NADH: ubiquinone oxidoreductase (complex I). *Journal of molecular biology*, *276*(1), 105-112.

36-Nelson DL, Cox MM. Lehninger principles of biochemistry. 6th ed. New York: W.H. Freeman; 2013. 1198p. https://www.whfreeman.com/lehninger6e

37-Berrisford, J. M., Baradaran, R., & Sazanov, L. A. (2016). Structure of bacterial respiratory complex I. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1857*(7), 892-901.

38-Guerrero-Castillo, S., Cabrera-Orefice, A., Huynen, M. A., & Arnold, S. Identification and evolutionary analysis of tissue-specific isoforms of mitochondrial complex I subunit NDUFV3. (BBA)-Bioenergetics, 2017. 1858(3), 208-217. https://doi.org/10.1016/j.bbabio.2016.12.004

39-Mckenzie, M., & Ryan, M. T. Assembly factors of human mitochondrial complex I and their defects in disease. IUBMB life, 2010. 62(7), 497-502. https://doi.org/10.1002/iub.335.

40-Opperdoes, F. R., & Michels, P. A. (2008). Complex I of Trypanosomatidae: does it exist?. *Trends in parasitology*, *24*(7), 310-317.

41- Bonelli P, Dei Giudici S, Peruzzu A, Mura L, Santucciu C, Maestrale C, et al. Identification of Echinococcus granulosus Genotypes G1 and G3 by SNPs Genotyping Assays. Pathog. 2021; 10(2): 1-10. https://doi.org/10.3390/pathogens10020125.

42-Korhonen PK, Kinkar L, Young ND, et al. Chromosome-scale Echinococcus granulosus (genotype G1) genome reveals the Eg95 gene family and conservation of the EG95-vaccine molecule. Commun biol 2022;5(1):199.

43-Rojas, C. A. A., Romig, T., & Lightowlers, M. W. (2014). Echinococcus granulosus sensu lato genotypes infecting humans–review of current knowledge. *International Journal for Parasitology*, *44*(1), 9-18.

44-Thompson, R. A. (2020). The molecular epidemiology of Echinococcus infections. *Pathogens*, *9*(6), 453.