Iraqi Journal of Veterinary Sciences

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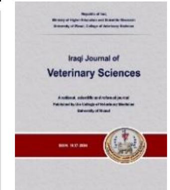

Hydatid cysts strains identification by mitochondrial dehydrogenase NADH subunit 5 isolated from cattle and buffalo host in Babylon governorate, Iraq

A.T. Farhood[1], A.I. Abdulzahra[2] and A.I. Saood[3]

[1]Department of Pathological Analysis, College of Science, University of Thi-Qar, Thi-Qar, [2]Department of Science, College of Basic Education, University of Babylon, Babylon, [3]Department of Diseases and Poultry Diseases, College of Veterinary Medicine, University of Basrah, Basrah, Iraq

Article information

Article history:
Received 15 August, 2023
Accepted 10 October, 2023
Published online 09 February, 2024

Keywords:
Echinococcus granulosus
Echinococcosis
NADH dehydrogenase subunit 5
Babylon

Correspondence:
A.I. Abdulzahra
bsc.st.amer.ebadi@uobabylon.edu.iq

Abstract

Hydatid cysts represent the larval stage of *Echinococcus granulosus* tapeworm and cause echinococcosis (Hydatidosis)in herbivores. It is a zoonotic disease that is widespread in the world. This parasite has Ten genotypes (G1-G10). The current investigation was carried out to identify the prevalent genotypes of *Echinococcus granulosus* in cattle and buffalo in Babylon governorate, Iraq. Forty hydatid cysts of the livers and lungs of cattle (25) and buffalo (15) were collected from the slaughterhouse of this governorate from March to October 2022. Cyst fluid was analyzed under a light microscope, and an eosin aqueous stain was used to determine the fertility based on the presence of protoscolices. Due to their high DNA purity and fertility of Hydatid cysts, 12 samples (6 include 3livers and 3lungs of cattle, were chosen (6 including three livers and three lungs' buffalo). Each sample's mitochondrial dehydrogenase NADH subunit 5 (NAD5) was amplified by 297 bp using molecular techniques (conventional PCR method). Isolate samples were recorded at Accession No. (LC775105- LC775116) in the Genbank. The results designate that the sheep strain (G1) buffalo (G3) was responsible for the hydatid cyst infection in Babylon governorate in 91.6 and 8.4%, respectively. Moreover, their sequences correlated with local and regional sequences and genetic diversity compared to the reference strains.

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Introduction

The most dangerous tapeworm infection that affects human health is *Echinococcus*, which causes hydatid disease and is caused by *E. granulosus* larvae (1,2). The disease is economically significant in terms of morbidity and mortality (3), especially in light of the discovery of several bacterial contaminations in hydatid cysts (4). Domestic animals are one of the many intermediate host species that the larvae of this worm can infect. The adaptation has made it available everywhere (5). This parasite has a complicated life cycle. The hydatid cysts HC are found in most organs of herbivores, such as liver, lung, spleen, brain, etc., while the mature worm is attached to the intestine of the Canidae family as a final host (6). The infection of HC leads to a disorder in the functions of the intermediate host organs (7). Consequently, it had a significant impact on the global economy (8). Genetic diversity may affect several different phenotypic characteristics in *E. granulosus*, such as host type, maturity rate, antigenicity, and spatial distribution (9). According to complete mitochondrial DNA sequencing, there are ten separate genotypes (G1- G10) of *E. granulosus sensu lato* (10). The most common strain is *E. granulosus sensu stricto* (G1-G3), found worldwide (11). Many other parasitological studies have been conducted in this governorate, such as studies of (12-16), but few on the *E. granulosus* parasite.

While They implemented many local studies to detect the types and strains of hydatid cysts in other governorates, The usage of *cox1* and *nad1* was most common (17-23). The *cox1* and *nad1* are not insufficient to designate genotypes (6), while Kinkar *et al.* (24) refer to the *nad5* gene region alone as giving enough data to distinguish between genotypes.

So, this study was designed to determine causative strain for hydatid cysts isolated from (the livers and lungs) of cows and buffaloes in Babylon governorate, based on primer (mt NAD5). A few studies used this gene in Iraq in general, and it was not used in a previous study in Babylon governorate, Iraq.

Materials and methods

Ethical approve

The approval was given to conduct this scientific work by the University of Thi-Qar, College of Science in their book No. 3/7/2854 on 6/3/2022.

Samples collection

Through the period from March to October 2022, collection of a total of 40 samples of hydatid cysts were collected. Obtained from Babylon governorate slaughterhouses as shown in (Table 1), the contents of hydatid cysts of chosen samples were centrifuged four times with a power of 300/ 10 minutes, each time using normal saline, and cyst fluid was examined under a microscope to determine the fertility based to presence protoscolices (25,26). After that, the sediments of the hydatid cyst were kept at freezing until the DNA extraction process (27).

DNA extraction

Following the instructions on the kit's purification, DNA was extracted from hydatid cysts and then frozen for later use.

Table 1: The number of infected Hydatid cysts is based on host, organ, and fertility

	Host	Organ	Fertility	Sample (n)	Host	Organ	Fertility
1	Cattle	Liver	Fertile	21	Cattle	Lung	Sterile
2	Cattle	Liver	Fertile	22	Cattle	Lung	Sterile
3	Cattle	Liver	Fertile	23	Cattle	Lung	Sterile
4	Cattle	Liver	Fertile	24	Cattle	Lung	Sterile
5	Cattle	Liver	Fertile	25	Cattle	Lung	Sterile
6	Cattle	Liver	Fertile	26	Buffalo	Liver	Fertile
7	Cattle	Liver	Fertile	27	Buffalo	Liver	Fertile
8	Cattle	Liver	Sterile	28	Buffalo	Liver	Fertile
9	Cattle	Liver	Sterile	29	Buffalo	Liver	Fertile
10	Cattle	Liver	Sterile	30	Buffalo	Liver	Sterile
11	Cattle	Liver	Sterile	31	Buffalo	Liver	Sterile
12	Cattle	Liver	Sterile	32	Buffalo	Liver	Sterile
13	Cattle	Liver	Sterile	33	Buffalo	Liver	Sterile
14	Cattle	Liver	Sterile	34	Buffalo	Liver	Sterile
15	Cattle	Liver	Sterile	35	Buffalo	Lung	Fertile
16	Cattle	Lung	Sterile	36	Buffalo	Lung	Fertile
17	Cattle	Lung	Sterile	37	Buffalo	Lung	Fertile
18	Cattle	Lung	Sterile	38	Buffalo	Lung	Sterile
19	Cattle	Lung	Sterile	39	Buffalo	Lung	Sterile
20	Cattle	Lung	Sterile	40	Buffalo	Lung	Sterile

Technique of polymerase chain reaction (PCR)

Forward and reverse primers were employed: F5′-GCC CCI ACI CCA GTI AGT TCT-3′ R5′-AAI ACA CTT AGA IAC ICC ATG ACT-3′ with molecular weight 297 bp, which were designed by Roelfsema *et al*. (28). All samples of previously extracted DNA were amplified using the traditional PCR technique. The final PCR reaction volume was 25 µl, which was made up of 0.5 µl of forward and reverse primers, 14 µl of nuclease-free water, five µl Master mix, and five µl of DNA. The following steps were used to carry out the reaction in a thermocycler (Table 2). DNA samples were put into the suitable wells of the TAE agarose gel 1.5% (w/v) and were dyed with 2µl of ethidium bromide dye. For 90 minutes at 70V, the agarose gel was run. Using a UV transilluminator, the DNA was visualized within an agarose gel. The length of the product of mt *nad5* was approximately 297 bp, and 100 bp was the size marker on the gels.

DNA Sequencing

For the purified PCR products obtained by the Geneaid DNA Cleanup kit, as described above, a tool named Bioedit

sequence alignment editor v.7.2.5 was used to fix the genetic data. The samples' hydatid cyst sequences were added to the NCBI database and assigned accession numbers (https://www.ncbi.n.lmnih.gov/).

Phylogenetic tree

The Maximum Composite of Likelihood method used genetic distance, UPGMA, and MEGA-X software v.11 for Phylogenetic tree analysis (29).

Table 2: PCR condition for *NAD5* gene

Steps	°C	Time	
Pre-Denaturation	94°C	5 minutes	
Denaturation	94°C	30 seconds	40 cycles
Annealing	55°C	30 seconds	
Extension	72°C	1 minute	
Final extension	72°C	5 minutes	

Results

Mitochondrial NAD5 Gene

Using *mt nad5* primer, we examine all chosen samples (12 samples), successfully ~ 297 bp (Figure 1).

For all the chosen 12 isolates, the partial sequencing of mt nad*5* produced a sequence of ~ 297 bp. The percentage of the sequence is identical between the study samples and other samples in NCBI-BLAST, ranging from 100%-95%. The sequences obtained have been deposited in the Genbank database under these accession numbers: LC775105, LC775106, LC775107, LC775108, LC775109, LC775110, LC775111, LC775112, LC775113, LC775114, LC775115, LC775116. Shows that G1 is the most prevalent and is followed by the G3 (Table 3).

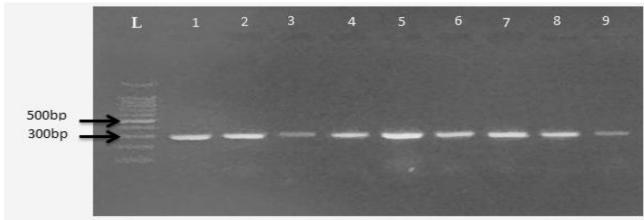

Figure 1: Agarose gel electrophoresis of PCR amplification *NAD5*. (L: 100bp DNA ladder, lanes 1-9: contain PCR products derived from: livers lungs of Cattle in lanes 1- 4, buffalo in lanes 5- 9 represent).

Phylogenetic analysis

Figure 2 showed evolutionary relationships between the mt *nad5* sequences produced in this study and the sequences found in Genbank of the *E. granulosus* strains and also explained that samples of this study have genetic diversity compared to the reference strains in Genbank. Their sequences showed a correlation with local and regional sequences.

Table 3: Genotypes of *Echinococcus granulosus*, its accession number, and identity ratio with other isolates for samples isolated from either livers and lungs Cattle and Buffalo based on the mt *nad5* gene and NCBI- BLAST alignment tool

Haplotype	Host	Organ	Accession No.	Identity (%)	Genotype	Accession No.	Country
1	Cattle	Liver	LC775105	99%	G1	MN199127.1	Nigeria
2	Cattle	Liver	LC775106	%96	G1	ON630427.1	Italy
3	Cattle	Liver	LC775107	%97	G1	MN270007.1	China
4	Cattle	Lung	LC775108	%99	G1	LC733547.1	Iraq
5	Cattle	Lung	LC775109	%97	G3	MG682544.1	Estonia
6	Cattle	Lung	LC775110	95%	G1	MK774655.1	Australia
7	Buffalo	Liver	LC775111	100%	G1	LC733543.1	Iraq
8	Buffalo	Liver	LC775112	99%	G1	LC733543.1	Iraq
9	Buffalo	Liver	LC775113	%98	G1	MK682656.1	Italy
10	Buffalo	Lung	LC775114	%97	G1	LC733547.1	Iraq
11	Buffalo	Lung	LC775115	%99	G1	MN270002.1	China
12	Buffalo	Lung	LC775116	99%	G1	LC733547.1	Iraq

Discussion

Genotyping is the essential and first step for detection, controlling the risk of spreading *E. granulosus* parasite, thus decreasing the infection. The result of the current study, depending on gene sequence analysis mt *nad5* 297 bp, showed only two genotypes G1 and G3. The sheep strain G1 was the dominant 91.6%. Eleven samples were liver and lung cattle and buffalo, whereas only 8.6 % were buffalo G3, represented by one sample only, Accession No. LC775109.

The previous studies in different provinces of Iraq recorded the same result but have used all *cox1*, *nad1*, *atp6*, or one of them, such as study Hammad *et al*. (30) recorded and G3 strains only in Kirkuk and Sulaimania, also in Thi-Qar and Misan governorates (31-33). However, these results differed from the studies of Hamoo *et al*. (34), Abdulla *et al*.

(35), were recorded G1 strain only in Aqrah and Koya cities in Dohuk and Erbil, northern Iraq, respectively, likewise recorded the same strain in other studies (36-38) from different governorates in middle Iraq and Kirkuk governorate respectively. Also disagreed with the studies of Al-Shimary (39), Al-Yaqoubi (40) in Al-Najaf and Al-Qadisiyah governorates, and Thi-Qar governorate respectively that recorded four genotypes together in addition to G1, G3, also recorded G2, G5. The results of this study may differ from previous studies due to different genetically contaminated ovules of study areas, or some studies use only the forward or reverse primer, and this produces an incomplete sequence for gene, compared with those used together so that the diagnosis may be inaccurate in these studies.

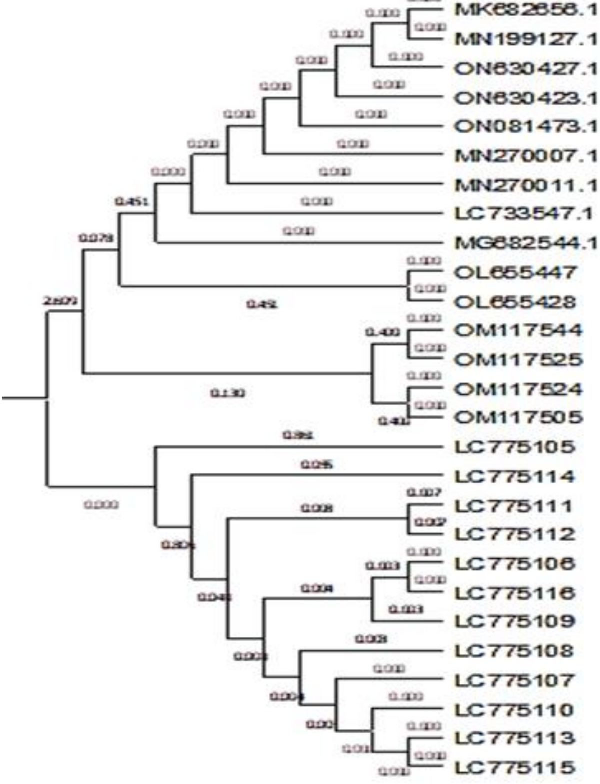

Figure 2: A Phylogenetic tree analysis based on mt *nad5* gene partial sequence used for *E. granulosus* genotyping among this study's isolates and other isolations deposited in the GenBank database. The phylogenetic tree was constructed using the Unweight Pair Group method with Arithmetic Mean (UPGMA tree) in MEGA-X software v.11).

These results prove that G1 is the most infected strain of Echinococcus spp. in Iraq. This might be due to its large capacity to infect several herbivorous hosts (8). The strains recorded diverse haplotypes associated with the local and global strains, although the dominating G1 strain. This genetic diversity in the study area may result from the interaction between wildlife hosts and herbivores (41-44). Additionally, the definitive host might be infected by various genetically mature worms that reproduce by various fertilization as cross and self-fertilization, thus producing a variety of ovules; therefore, produced hydatid cysts are different genetically (45-47).

Conclusion

There is genetic diversity for haplotypes in G1 that recorded dominance strain, followed by G3 of larval *E. granulosus* (hydatid cysts) in Babylon province. Different genotypes of hydatid cysts may not have been the source of intermediate hosts. The mt NAD5 primer is reliable for identifying the parasite strains and can be used in future studies.

Acknowledgments

The author thanks the veterinarian employees at the Babylon governorate slaughterhouse, especially Dr. Aqeel Mohammed and Dr. Ahmed Ali, for helping us collect samples in this study.

Conflict of interest

The authors' interests do not conflict with this paper's publication.

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تحديد سلالات الأكياس العدرية بالاعتماد على نازعة ثنائي نوكليوتيد الأدنين وأميد النيكوتين الوحدة الفرعية الخامسة المايتوكوندري المعزولة من الأبقار والجاموس في محافظة بابل، العراق

آفاق طالب فرهود[١] أمير إبراهيم عبد الزهره[٢] و علاء إسماعيل سعود[٣]

[١]قسم التحليلات المرضية، كلية العلوم، جامعة ذي قار، ذي قار، [٢]قسم العلوم، كلية التربية الأساسية، جامعة بابل، بابل، [٣]قسم الأمراض وأمراض الدواجن، كلية الطب البيطري، جامعة البصرة، البصرة، العراق

الخلاصة

الأكياس العدرية تمثل المرحلة اليرقية لدودة المشوكة الحبيبية الشريطية وتسبب مرض المشوكات الحبيبية (الأكياس العدرية) في الحيوانات العواشب (الحيوانات المستأنسة) ويعد من الأمراض الحيوانية المنشأ المنتشرة في العالم، المشوكة الحبيبية تضم عشرة أنماط وراثية (النوع ١- النوع ١٠). أجريت هذه الدراسة لتحديد الانماط الوراثية الشائعة المسببة للأكياس العدرية في بعض المضائف الوسطية (الابقار والجاموس) في محافظة بابل، العراق. تم جمع أربعين كيسا عدرياً لأكباد (٢٥) ورئات (١٥) من الأبقار الجاموس على التوالي من مجزرة هذه المحافظة، خلال المدة من نيسان إلى تشرين الأول لعام ٢٠٢٢. حددت الخصوبة لسائل الأكياس العدرية على أساس وجود الرؤيسات الاولية عن طريق الفحص المجهري واستخدام صبغه الأيوسين المائية، اختيرت ١٢ عينة (٦ أبقار تتضمن ٣ أكباد و٣ رئات) و (٦ جاموس تتضمن ٣ أكباد و٣ رئات) بالاعتماد على نقاوة الحامض النووي وخصوبة الأكياس العدرية واستخدمت طريقة تفاعل البلمرة المتسلسلة التقليدية لتحديد جين ثنائي نوكليوتيد الأدنين وأميد النيكوتين الوحدة الفرعية الخامسة بوزن ٢٩٧ لكل قاعدة. سجلت العينات المعزولة في بنك الجينات العالمي بأرقام تتراوح بين (LC775105- LC775116). أشارت النتائج إلى أن سلالة الأغنام (النوع ١) وسلالة الجاموس (النوع الثالث) هي مسؤولة عن الإصابة في الأكياس العدرية في محافظة بابل بنسب ٩١,٦ و ٨,٤%، على التوالي. بالإضافة إلى أن تسلسلات هذه السلالات أظهرت ارتباطها مع تسلسلات محلية وإقليمية، فضلا عن التنوع الجيني مقارنة مع السلالات المرجعية المسجلة في بنك الجينات العالمي.