Determination the causative strain for hydatid cyst in Iraqi cattle by using *ND1* gene

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Summary

Hydatid Cysts were obtained from 15 cows from liver, lung, spleen, heart, and peritoneal cavity, between December 2014 and October 2015. Hydatid cysts (protoscoleces) were used for deoxyribonucleic acid extraction by using mechanical grinder. The purification of mtDNA was done by (promega kit, USA). The mitochondrial NADH dehydrogenase subunit 1 (*ND1*) genes were used as targets for polymerase chain reaction amplification, all hydatid cysts yielded amplification products. Polymerase chain reaction product for NADH1 800 basic pair. The polymerase chain reaction products were purified and partial sequences were generated. The sequences obtained were found to align with corresponding region for *ND1* gene in the Gene Bank nucleotide database confirming to genotype of sheep strain (G1) in Iraq, Phylogenetic analysis of partial sequence data from *ND1* genes for obtained Phylogenetic tree. G1 genotype was the most common taxon and was the actual source of infection of Iraqi's cattle. All of 15 strains were G1 genotype (sheep strain) based on the partial sequences of NADH dehydrogenase 1 (*ND1*).
Keywords: G1 strain hydatid cyst, *ND1* gene, Cattle, Sequences.

Introduction

The larval stage caused Cystic echinococcosis of tape worm *Echinococcus granulosus* has a cosmopolitan distribution and is one of the most significant zoonosis all around the world (1 and 2). Cattle acquire infection by coming in contact with infected dogs harboring adult *E. granulosus* in their intestine resulting in excretion of eggs in the feces. Thus, one way that humans and other intermediate host can be infected is by swallowing ova that contaminate food, water or the environment generally (3). The extensive variation in *E. granulosus* may affect life cycle, host specificity, rate of development, pathology and consequently for design plus sensitivity to chemotherapeutic agents and development of vaccines against *E. granulosus* (4). In Iraq, CE constitutes one of the major endemic diseases and its seriousness in both humans and animals which has serious impacts on human or animal health (5-10). To date, 10 distinct genotypes (G1-G10 strain) have been described in the world based on nucleotide sequences analysis of the (*CO1*), (*ND1*) genes and intra transcribed spacer 1 (ITS1); these genotypes have been associated with distinct, intermediate hosts: sheep, goats, pigs, cattle, horses, camels and cervides (11-16). The G1 genotype was also identified as the only genotype present in isolated from cattle, sheep and humans (17). A number of deoxyribonucleic acid (DNA) identification techniques have been employed to better identified *Echinococcus* species and genotypes from different intermediate and definitive hosts and in different geographical settings (18).

The aim of this study was to characterize the *E. granulosus* genotypes currently infecting cattle in Iraq, using polymerase chain reaction (PCR) and to estimate the genetic variability within the strains by sequencing the NADH dehydrogenase subunit 1 (ND1) genes.

Materials and Methods

The contents of 15 hydatid cysts were collected from different region of Iraq during 2014 to 2015. Once cysts were washed several times with normal saline to decrease contamination with host tissue, and then they were extensively washed with 70% ethanol. According to (19), each cyst was separated into membrane and intra cystic fluid with protoscoleces. The cyst contents (fluid and protoscoleces) were aspirated aseptically by sterile syringes (10 ml) into flask. Cysts were

opened longitudinally incision and all the remaining protoscolex and fluid were aspirated and added to the flask content. The fluid was carefully and gradually decanted into sterile test tubes with spinning by centrifugation at 3000 rpm for l0 min. at room temperature to get of the protoscoleces to pellet. The germinal membrane was peeled away and washed several times with Hanks saline (pH 2.0) containing 0.2% (w/v) pepsin to free the remaining attached scoleces (20). The suspension was centrifugated at 3000 rpm for 10 min., and the pellet of scoleces was collected. Protoscoleces were finally rinsed 3-4 times with sterile normal saline by repeated centrifugation followed by 70% ethanol, and stored in 70% ethanol at 4 ^{o}C temperature for further analysis. Pellet of protoscoleces were rinsed several times with sterile distilled water and Phosphate buffer saline (PBS) to remove ethanol prior to DNA extraction (21). DNA extraction was done by using Wizard ®Genomic DNA Purification Kit. (USA) and follow the instruction of manufacture (22). 20 ng of DNA from pellet of protoscoleces was used in all samples.

Twenty ng of DNA samples were analyzed by using the described methods with minor modifications (22) (Table, 1). The mitochondrial *ND1* region was amplified by PCR using *ND1* F. and *ND1* R. primers (Table, 2) (17). The thermal conditions of the PCR *ND1* reaction were as follows, denaturation for 4 min. at 94ºC, followed by 35 cycles of 45 second at 94ºC, 45 second at 58ºC and 45 second at 72ºC and a final extension at 72ºC for 7 min.

Table, 1: PCR condition for *ND1* gene.

Criteria of cycle	Conditions (temp/ time)
Initial denaturation	94°C/5 min.
Amplification	94°C/45 s; 58°C/45 s; 72°C/45 s
Number of cycles	35
Final extension	72°C/7 min.

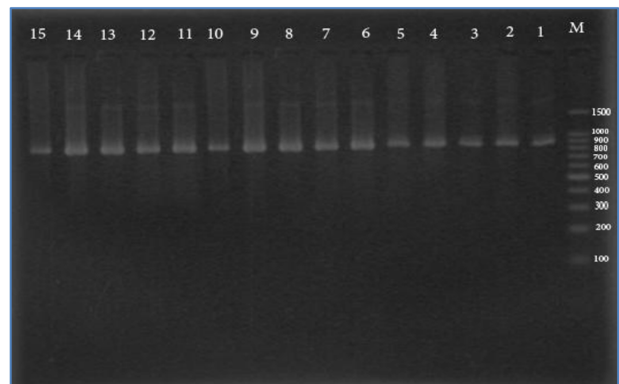
$_{mt}$DNA sequencing and phylogenetic analysis fifteen amplicons were selected, and fragments of amplicons *ND1* genes were amplified with primers published (17). DNA sequences were compared with partial *ND1* sequences from previous publications and NCBI website (http://www.ncbi.nlm.nih.gov). Phylogenetic analysis of partial sequence data from *ND1* genes.

Table, 2: NADH dehydrogenase subunit 1 ND1 primer.

Marker	Size	Code	Sequence
ND1	800 bp	*ND1*.F	5'-GTT TTT GGG TTA GTC TCT GG-3'
		ND1.R	5'-ATC ATA ACG AAC ACG TGG -3'

Results and Discussion

The target DNA was successfully amplified from 15 hydatid cyst isolates were prepared for PCR process by using specific primer. A PCR process to amplify DNA as the target gene was set up and performed on all 15 DNA samples. The agarose gel electrophoresis of PCR amplicon of *ND1* gene (Fig.1).



Figure, 1: Shows the agarose gel electrophoresis of PCR amplicon of *ND1* gene, using 2% agarose, 80 V, 70 Am for 2 hrs., (lanes 1-15: *E. granulosus* isolates; M: 100 bp DNA ladder).

A partial region of the *ND1* gene was amplified following a previously described protocol. To determine the genotypes of 15 isolates of cysts, NADH dehydrogenase subunit 1 gene was amplified by PCR, then sequenced and analyzed by alignments with reported reference sequences of G1 genotype of *E. granulosus* using Gene bank (Fig. 2). The sequence alignment was done by using Bioedit (DNA analysis program) and compared with previously reported references of *E. granulosus* genotypes. The results revealed 100% were identical with common sheep strain G1 genotype comparing with [ACCESSION KU925430] (23).

Score	Expect	Identities	Gaps	Strand
1410 bits(763)	0.0	763/763(100%)	0/763(0%)	Plus/Plus

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Query  20    GTTTGTTAATAATTGCCTTTTTTGTTTTAGGGGAGCGTAAGGTTTTGGGCTATTCTCAGT   79
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4480  GTTTGTTAATAATTGCCTTTTTTGTTTTAGGGGAGCGTAAGGTTTTGGGCTATTCTCAGT   4539

Query  80    CTCGTAAGGGCCCTAACAAGGTTGGTGTAATTGGTTTGTTGCAGAGGTTTGCTGATCTAT   139
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4540  CTCGTAAGGGCCCTAACAAGGTTGGTGTAATTGGTTTGTTGCAGAGGTTTGCTGATCTAT   4599

Query  140   TGAAGTTGGTAATTAAGTTTAAGTGTTTTTACTTCCAAAGTCGTAGGTATGTTGGTTTGT   199
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4600  TGAAGTTGGTAATTAAGTTTAAGTGTTTTTACTTCCAAAGTCGTAGGTATGTTGGTTTGT   4659

Query  200   TTGGTGTTGTGTTATTAATGGCTTTGGTGATTGTTTATTCATTTATTTATGGTAGATATT   259
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4660  TTGGTGTTGTGTTATTAATGGCTTTGGTGATTGTTTATTCATTTATTTATGGTAGATATT   4719

Query  260   ATAGAGCTAGTTATAGAGGCCTCTCCGTGTTGTGGTTTTTGGCTGCCGCCAGAACATCTA   319
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4720  ATAGAGCTAGTTATAGAGGCCTCTCCGTGTTGTGGTTTTTGGCTGCCGCCAGAACATCTA   4779

Query  320   GGTATTCTTTGTTGTGTACTGGTTGGGGTGGTTACAACAATTATTCATTTTTAAGGTCGG   379
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4780  GGTATTCTTTGTTGTGTACTGGTTGGGGTGGTTACAACAATTATTCATTTTTAAGGTCGG   4839

Query  380   TTCGATGTGCTTTTGGATCTGTTAGGTTTGAGGCTTGTTTTATGTGTGTGGTGATTTTTT   439
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4840  TTCGATGTGCTTTTGGATCTGTTAGGTTTGAGGCTTGTTTTATGTGTGTGGTGATTTTTT   4899

Query  440   GTGCTTTGTGTAGTTGTAGGtataatttaattgatttttattataattgttgattaagtt   499
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4900  GTGCTTTGTGTAGTTGTAGGTATAATTTAATTGATTTTTATTATAATTGTTGATTAAGTT   4959

Query  500   tgttattatttccattaatttatgtgttatttttaatatgtATATTGTGTGAAACTAATC   559
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4960  TGTTATTATTTCCATTAATTTATGTGTTATTTTTAATATGTATATTGTGTGAAACTAATC   5019

Query  560   GTACGCCATTTGATTATGGAGAGGCTGAAAGAGAGTTGGTCAGTGGGTTTAAAGTTGAGT   619
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5020  GTACGCCATTTGATTATGGAGAGGCTGAAAGAGAGTTGGTCAGTGGGTTTAAAGTTGAGT   5079

Query  620   ATAGTGGTATTTATTTTACGTGTTTATTTGCTTGTGAGTATATTATTATATATGTGTTTT   679
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5080  ATAGTGGTATTTATTTTACGTGTTTATTTGCTTGTGAGTATATTATTATATATGTGTTTT   5139

Query  680   CATGGTTGGGAGTTGTGTTGATGTTTGGTGGCGGTTTTATCGGTATGTTGGTGTTAGTGT   739
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5140  CATGGTTGGGAGTTGTGTTGATGTTTGGTGGCGGTTTTATCGGTATGTTGGTGTTAGTGT   5199

Query  740   TTAATTTATTATTTTTTTATGTGGGCTCGGGCGACATTACCACG                 782
             |||||||||||||||||||||||||||||||||||||||||||
Sbjct  5200  TTAATTTATTATTTTTTTATGTGGGCTCGGGCGACATTACCACG                 5242
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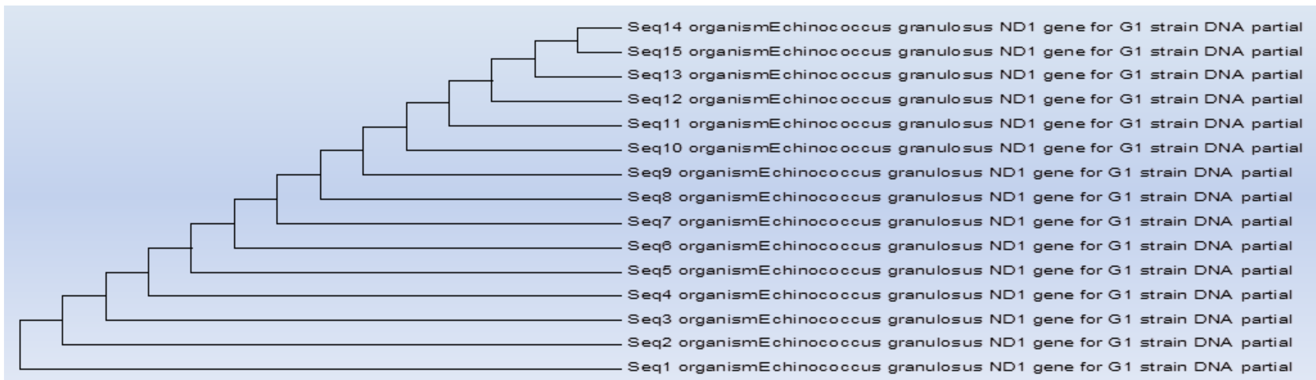
Figure, 2: Alignment of *NAD1* gene with reported reference sequences of G1 genotype of *E. granulosus* by using Gene bank.

The *ND1* region was amplified by using a primer set designed for this study. The PCR amplification of *ND1* gene was successful in all isolates and generated products of approximately 800 bp, this result agreed with previous results done by (17, 24 and 25) where showed the size of NADH dehydrogenase subunit 1 (*ND1*) was 800 bp. Genotype G1 was the most common sheep infectious *E. granulosus* genotype in the world with a wide range of hosts (26). In these regions, dogs are often feed with livestock viscera that may be infected with the parasite (16). This activity could be sufficient to propagate the current endemic state. From the results above, it can be indicated that the prominent circulation of the common sheep genotype (G1) was prevalent in hyper-endemic areas of Iraq. This result agreed with (16, 25 and 26) possibly because it is the most common one also it is widespread among intermediate hosts (17 and 27). The identification of the genotypes of *E. granulosus* present in livestock and wild animals within regions endemic for *E. granulosus* is epidemiologically important. G1 genotype

strains may also infect other intermediate host such as goats (28 and 29). A phylogenetic tree of the *ND1* genotypes was constructed using the maximum likelihood calculates with the Tamura-Nei distance, as the model of evolution (Fig. 3), (30).



Figure, 3: Molecular phylogenetic analysis by maximum likelihood method

The evolutionary history was inferred by using the ceiling Likelihood method based on the Tamura-Nei model (30). The tree with the highest log likelihood (-980.0307) is shown. Elementary tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a template of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with officer log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of replacement per site. The test involved 15 nucleotide sequences. Codon positions included were 1st $+2^{nd}$ $+3^{rd}$ + Noncoding. All positions including gaps and absent data were eliminated. There were a total of 783 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (31). In conclusion the G1 genotype was the most common taxon and the actual source of infection of Iraqi's cattle. All of 15 strains were G1 genotype (sheep strain) based on the partial sequences of NADH dehydrogenase 1 (*ND1*).

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تحديد السلالة المسببة للأكياس المائية في الأبقار العراقية باستعمال جين *ND1*

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الخلاصة

إستُحصلت الأكياس المائية من 15 بقرة من الكبد، الرئة، الطحال، القلب والتجويف البريتوني ما بين كانون الأول 2014 وتشرين الاول 2015 وقد استعملت رويسات الأكياس المائية لاستخلاص الدنا باستخدام طريقة السحق الميكانيكي. نُقّيَ الدنا المايتوكونديري باستعمال كت (بروميكا. امريكي المنشأ). وقد استعمل جين ND1 كهدف لتفاعل سلسلة البوليمرات وقد أعطت جميع الأكياس المائية نتائج للتضاعف وكان حجم الجين 800 زوج قاعدي، نُقّيَ ناتج التضاعف وحُدد تسلسل القواعد النايتروجينية له. اجريت مطابقة للتسلسلات التي إستُحصل عليها في بنك الجينات العالمي لإثبات الإصابة بسلالة الاغنام G1 في العراق. وقد أجري تحليل للسلالات الوراثية للجين للحصول على الشجرة الوراثية. النمط الوراثي G1 اكثر الاصناف انتشاراً ويعتبر مصدر الاصابة للابقار العراقية. جميع الـ15 سلالة كانت للنمط الوراثي G1 (سلالة الأغنام) اعتماداً على التسلسلات الجزئية لـ NADH.

الكلمات المفتاحية: سلالة الأكياس المائية، جينND1، الأبقار، تسلسلات.