

Prevalence and molecular studies on *Echinococcus equinus* isolated from necropsied donkeys

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Abstract

In the present study, forty donkeys of different ages and sexes at Giza Zoo, Egypt were investigated between October 2015 and September 2016 for the presence of hydatidosis disease. Hydatid cysts were detected in the livers of 10% of the examined donkeys and these cysts had a fertility rate 100%. Female donkeys were infected with cysts more than males and all infected donkeys were old aged with no cases of infection were detected in young or adult donkeys. Using molecular tools, the DNA extracted from cysts that had been isolated was subjected to PCR amplification, using synthesized oligonucleotide primers, and these were constructed to target the 299 bp within the (ND2) gene, which is considered to be specific for the *Echinococcus equinus* genotype. The sequenced PCR products showed homology to *E. equinus* (G4 or horse strain genotype). These results can be used in future to pursue the epidemiological status of the causative strain of hydatidosis in equines at the study area.

Keywords: hydatidosis, PCR, horse strain, equines

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دراسة عن الإنتشار والتشخيص الجزيئي لطفيل *Echinococcus equinus* المعزول من الحمير المشرحة

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الخلاصة

في هذه الدراسة، تم فحص عدد أربعين حماراً من مختلف الأعمار والأجناس في حديقة حيوانات الجيزة، مصر في الفترة بين أكتوبر 2015 وسبتمبر 2016 لوجود مرض الأكياس المائية. تم الكشف عن الأكياس المائية لمرض الأكياس المائية في كبد 10% من الحمير المفحوصة و كان معدل الخصوبة لتلك الأكياس 100%. أظهرت الحمير الإناث معدل إصابة أعلى من الذكور وكانت جميع الحمير المصابة من الأكبر سناً ولم يتم الكشف عن أي حالات إصابة في الحمير الصغيرة أو البالغة. باستخدام أدوات التشخيص الجزيئية، تم إخضاع الحامض النووي الديوكسي ريبوزي المستخرج من الأكياس المعزولة لتفاعل إنزيم البلمرة المتسلسل باستخدام بواقي تفاعل مخصصة لتضخيم 299 زوج قاعدي بداخل جين ND2 والخاصة بعنزة *Echinococcus equinus* و بإجراء إختبار التتابع الجيني أظهرت العينات المعزولة تطابقاً لعنزة *Echinococcus equinus*. يمكن استخدام هذه النتائج في المستقبل لمتابعة الحالة الوبائية للسلسلة المسببية لمرض الأكياس المائية في الخيول بمنطقة الدراسة.

Introduction

Hydatidosis is an important world-wide zoonotic disease caused by the larval stages (metacestodes) of

tapeworm parasites of the genus *Echinococcus*. These cestodes have an indirect, life cycle, with carnivores (canids) as definitive hosts and herbivorous or omnivorous mammals as intermediate hosts. Adult tapeworms inhabit

the small intestine of the definitive host and these release gravid segments containing eggs into the environment via feces. When these eggs are ingested by intermediate hosts, the latter become infected with larvae of *Echinococcus spp.*, fluid-filled hydatid cysts (HC) formed mainly in their livers and lungs, in which protoscoleces are produced as the next generation of tapeworms. Their growth can cause severe illness and death in the intermediate host (1). Infection with *E.granulosus* in intermediate hosts is typically asymptomatic, although there have been a few cases noted of long-standing, heavy infections (2).

The disease is endemic in many parts of the world. It is one of the major zoonotic parasitic diseases in the Middle East and Arab North Africa, from Morocco to Egypt (3). In Egypt, existing studies which have been carried out to determine the infection rate of HC among Egyptian equids showed that it was 4.62% in Qaliobia and Giza Governorates (4), 6.89% in Beni-Suef Governorate (5) and 14.2% in Giza Governorate (3). More widely, the infection rate of equids with HC in different countries was found to be 16.7% in Morocco (6), 0.27% in Italy (7), 8.9% in Chile (8), 12.2% in Romania (9) and 2% in Iran (10).

Evidence is being accumulated that genetic heterogeneity is common within the species of the genus *Echinococcus*. Thus, it may be said that a strain of *Echinococcus* can be defined as a group of individuals which differs statistically from other groups of the same species in gene frequencies. This has a significant impact on the epidemiology, pathogenicity and control of hydatid disease (11).

For identification of *E.granulosus* at the species level, the use of morphological and biological studies have provided extremely useful information (10) but it should be considered that these features are variable. They may be influenced by host and environmental factors and may not necessarily reflect distinctness at the genetic level. Molecular techniques allow a direct characterization of the genome of the parasite and these techniques have the advantage that they are not confounded by variability induced by the host or the environment (11,12).

Molecular genetic studies based on mitochondrial DNA analysis have demonstrated that *E. granulosus* is actually a complex of genotypes that exhibit a marked genetic variability. At least ten distinct genotypes (G1–G10) have been identified within the *E. granulosus* complex. These include two sheep strains (G1 and G2), two bovid strains (G3 and G5), a horse strain (G4), a camel strain (G6), two pig strains (G7 and G9) and cervid strains (G8 and G10) (5,13,14). *E.equinus* (also known as *E. granulosus G4* or *horse strain*) uses equids as specific intermediate hosts (1,5,7,15–21). No human cases of infection with *E.equinus* are known, and the epidemiological evidence suggests that it may be non-pathogenic to humans (13,15). Other

Echinococcus strains (*E. granulosus G1* or *sheep strain*) might also cause hydatidosis in equids (7,22).

The aim of this study is to genetically characterize the isolates of HC from donkeys examined in Giza zoo, in order to determine the causative strain of hydatidosis of donkeys in the study area.

Material and methods

Study area

This study was conducted from October 2015 to September 2016 in the abattoir of the Giza Zoo, Egypt. The animals that were examined were brought to the zoo from the centers and nearby cities of Cairo and Giza Governorates, where they were slaughtered to feed predators. The samples were collected from (40) donkeys of both sexes and of varying ages. Donkeys were grouped into three age groups according to (4) (Table 1).

Table 1: Number of donkeys examined, according to sex and age

Age groups	Sex	
	Males	Females
Young donkeys (<2 years)	2	1
Adult donkeys (≥2 or ≤10 years)	9	8
Old donkeys (>10 years)	7	13
Total (40)	18	22

Collection of parasite material

After slaughtering, each donkey was examined for the presence of HC, using palpation and incision (3). The intact cysts were removed from an infected animal, put in separate, polyethylene bags and labeled with the necessary data (age, sex, infected organ and date of collection), then sent in an ice box to the Biotechnology lab, Animal Health Research Institute, Dokki, for examination. In the lab, each cyst was washed with distilled water to remove debris, incised and then HC fluid was aspirated using a sterile syringe. The collected fluid was centrifuged at 3000 rpm for 7 minutes and the sediment was collected.

Checking the fertility of cysts

The sediment of each cyst was observed under a light microscope at 40x magnification. Cysts with no protoscoleces were considered to be sterile cysts (23). After being examined, the sediment was preserved in 70% ethyl alcohol for further use.

Molecular characterization of HC isolates

Parasite material

70% ethyl alcohol preserved HC sediments (containing protoscoleces) which were collected from fertile HC.

DNA extraction

DNA was extracted from preserved protoscolecuses using the Thermo Scientific GeneJET Genomic DNA Purification Kit according to manufacturer's instructions (Cat No #K0722).

Oligonucleotide primers

For conventional PCR, oligonucleotide primers were synthesized as follows:- forward:

5'-GGTTTGGAGATA CATAATAATGTCCGGAC-3'

and reverse:

5'-CTCACACCAAGCACCTACACATAAATATAGTT-3'

(20). These oligonucleotide primers were constructed to target 299 bp within the NADH (Nicotinamide Adenine Dinucleotide) dehydrogenase subunit 2 (ND2) gene, which is considered specific to the *E. equinus* genotype.

Polymerase Chain Reaction (PCR) amplification

DNA amplification was done in 20 µL reaction volume containing 4 µL 5 x FIREPol Master Mix, 0.5 µL (10 pmol/ µL) from each primer, 3 µL extracted DNA and then filled up to 20 µL with DNase/RNase free water. Negative control (no-DNA) reaction was included in PCR amplification. The amplification procedure was carried out using MJ Mini thermal cycler (Bio-Rad) with the following thermal profile: 95°C for 5 min as an initial denaturation, 35 cycles of 94°C/30s (denaturation step), 57°C/30s (annealing step), 72°C/30s (extension step) and a final

extension at 72°C for 5 min. 5 µL aliquots of PCR products were then fractionated on 1.5% agarose gel stained with ethidium bromide, which marked by 50 bp DNA ladder (Genedirex). The electric current was adjusted at 100 V for 30 minutes, and then gel was visualized and photographed using the Bio-Rad gel documentation system.

DNA sequencing

Samples were considered positive for *E. equinus* when a single band of DNA at 299 bp was evident in ethidium bromide agarose gel. The PCR amplicons (n=2), after being fractionated on ethidium bromide agarose gel, were cut from the gel and purified using the Thermo Scientific GeneJET Gel Extraction Kit (Cat No. K0691). The purified PCR products were then sequenced in one direction, using reverse primer. The sequencing procedure was done at the Genome unit, Animal Health Research Institute, Dokki.

Computer-assisted sequences and phylogenetic analysis

The electropherogram of each sequence (n=2) was checked by eye. The resulting nucleotide sequences were aligned and compared to each other using BioEdit software (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Using the Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov>), the DNA sequences obtained were subsequently aligned and compared with verified sequences of *E. equinus* and other *E. granulosus* strains available in the Genbank (Table 2).

Table 2: Genbank accession numbers of verified sequences of *Echinococcus equinus* and other *E. granulosus* strains used in analysis

Accession numbers	Genotype
KY766905.1	<i>Echinococcus equinus</i> isolate G4 mitochondrion partial genome
AB786665.1	<i>Echinococcus equinus</i> mitochondrial DNA complete genome sample code: EEQU/JEN
AF346403.1	<i>Echinococcus equinus</i> mitochondrion complete genome
AB235848.1	<i>Echinococcus canadensis</i> mitochondrial DNA complete genome genotype G8
AB208063.1	<i>Echinococcus canadensis</i> mitochondrial DNA complete genome
AB745463.1	<i>Echinococcus canadensis</i> mitochondrial DNA complete genome genotype: G10
AB786664.1	<i>Echinococcus granulosus</i> mitochondrial DNA complete genome sample code: 52LI07
AB208546.1	<i>Echinococcus vogeli</i> mitochondrial DNA complete genome
AF297617.1	<i>Echinococcus granulosus</i> genotype I mitochondrion complete genome
AB208064.1	<i>Echinococcus shiquicus</i> mitochondrial DNA complete genome
KC897684.1	<i>Echinococcus granulosus</i> isolate P NADH dehydrogenase subunit 2 (ND2) gene complete cds mitochondrial
KC897682.1	<i>Echinococcus granulosus</i> isolate N NADH dehydrogenase subunit 2 (ND2) gene complete cds mitochondrial
KU601616.1	<i>Echinococcus granulosus</i> genotype Omo mitochondrion complete genome
KY766901.1	<i>Echinococcus granulosus</i> isolate TUR1 mitochondrion partial genome
KY766900.1	<i>Echinococcus granulosus</i> isolate SPA1 mitochondrion partial genome
KY766894.1	<i>Echinococcus granulosus</i> isolate IRA1 mitochondrion partial genome
AB235846.1	<i>Echinococcus ortleppi</i> mitochondrial DNA complete genome genotype G5
AY684274.1	<i>Taenia saginata</i> mitochondrion complete genome

The phylogenetic tree was constructed by the software program Mega 7 (<http://www.megasoftware.net>), using the neighbor-joining method. *Taenia saginata* (AB684274.1) was used as an out group. The sequences analyzed in the present study were then deposited in the Genbank under accession numbers (MF442262 and MF442263).

Results

Out of 40 examined donkeys, 4 donkeys harbored HC. The infection rate was 10%. Female donkeys were harbored HC more than males (Table 3). All infected donkeys were old aged with no cases of infection were detected in young or adult donkeys. In all infected animals, the cysts were present only in the liver and there was no evidence of cyst formation in the lungs. The number of cysts observed in infected livers ranged from 2 to 4 cysts, and these were of different sizes. All the collected cysts were fertile and harbored protoscolices (Table 4).

On using extracted DNA as a template for PCR amplification, using synthesized oligonucleotide primers, the result showed 299 bp fragments in ethidium bromide agarose gel which were specific for *E. equinus* (Fig 1).

The alignment of partial nucleotide sequence of ND2 isolates obtained from examined donkeys showed that they had 100%, 90%, 90%, 89% and 92% homology to *E. equinus* (AB786665.1), *E. granulosus* (KJ559023.1) *E. canadensis* (AB208063.1), *E. vogeli* (AB208546.1) and *E. shiquicus* (AB20864.1) respectively. When the nucleotide sequences of isolates were aligned to the reference nucleotide sequence of *E. equinus* accessed in the Genbank they revealed insertion and deletion of nucleotides at several positions (Fig 2). The phylogenetic analysis showed that the obtained isolates belonged to *E. equinus* (Fig 3).

Table 3: Infection rate of HC in the examined donkeys

Sex	Number of the examined animals	Number of the infected animals
Males	18	1
Females	22	3
Total	40	4
Infection rate (%)	10%	

Table 4: Number, distribution and fertility of HC in infected donkeys

Donkey	Age	Sex	Recovered hydatid cysts (HC)							
			No. of cysts	Inf. organ	Fertile		Sterile		Calcified	
					No.	%	No.	%	No.	%
1	18 year	Female	2	Liver	2	100%	-	-	-	-
2	12 year	Female	3	Liver	3	100%	-	-	-	-
3	28 year	Female	2	Liver	2	100%	-	-	-	-
4	11 year	Male	4	Liver	4	100%	-	-	-	-

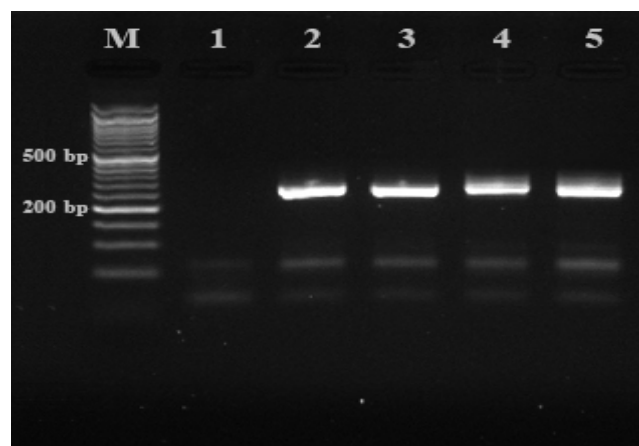


Figure 1: 1.5% ethidium bromide stained agarose gel electrophoresis. Lane M: 50 bp ladder marker. Lane 1: negative control. Lane 2-5: amplified fragments of 299 bp resulted from amplification of extracted DNA using synthesized oligonucleotide primers specific for *E. equinus*.

Discussion

In the present study, the infection rate of HC is (10%). This result is considered higher than results obtained by (4,5) while it is lower than the result obtained by (3), who carried out their necropsy examination in different localities in Egypt. The infection rate is higher than that obtained by (7,8,10) while it is lower than that obtained by (6,9) who carried out their studies in different parts of the world. The difference in infection rate may be due to variation in environmental factors such as temperature, humidity and the nature of the pasture (24).

All infected animals harbored HC only in their livers, a result that agrees with that obtained by (4,10) while it disagrees with results obtained by (5–8) who showed presence of HC in the liver and lung of infected animals, and (1) in Germany, who demonstrated the presence of HC only in the lung of infected mares. This may be due to the bile duct in the liver, which receives blood with oncospheres after it passes the duodenum (24).

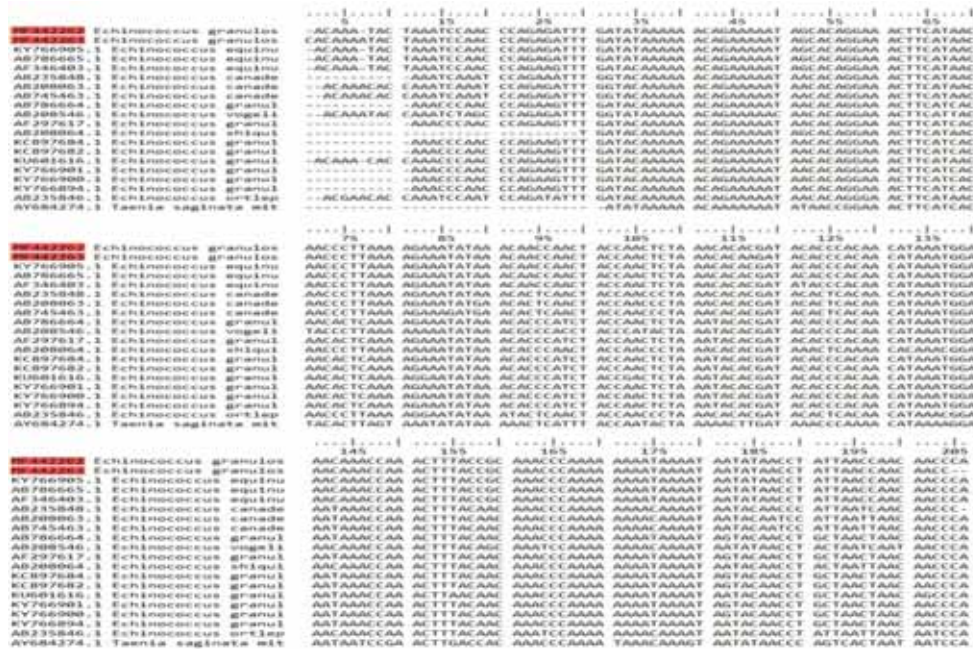


Figure 2: Alignment of nucleotide sequence of *E. equinus* of isolates obtained from donkeys examined at Giza Zoo, Egypt (labeled) with reference to sequences of *E. equinus* accessed in the genbank, in addition to sequences of different genotypes of genus *Echinococcus*.

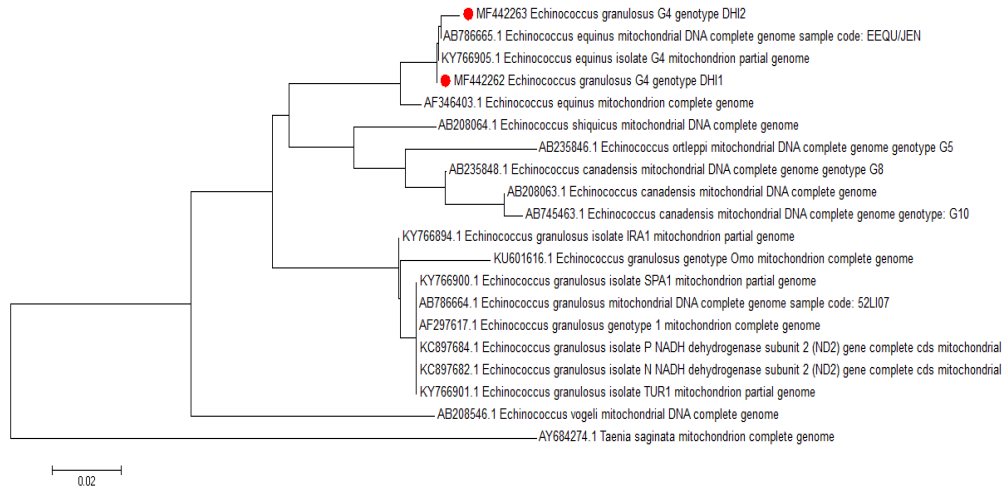


Figure 3: Genetic relationship of *E. equinus* isolates obtained from examined donkeys at Giza Zoo, Egypt (labeled) with reference to sequences of *E. equinus* accessed in the genbank and with reference to sequences of other genotypes of genus *Echinococcus* which were constructed by phylogenetic analysis of the nucleotide sequence of the ND2 gene.

The livers infected with HC in this study show a fertility rate (100%) which agrees with the result obtained by (10) and disagrees with the result obtained by (3,5,7). This may be because the causative strain (*E. equinus*) tends to produce very well-developed hydatid cysts (7).

The prevalence of HC according to sex showed that most cases of infection occurred in female donkeys; only

one case was diagnosed in male donkeys which agrees with the result obtained by (4,25). (26) Proved that infection with HC is equally prevalent in male and female donkeys. This may be because female donkeys have a long gestation period, and because of the adverse environmental conditions suffered by donkeys in the Middle East countries (25).

Our result showed further that the infections with HC occurred in older animals, and this disagrees with the results obtained by (4), who reported that HC infection occurs preferentially in adult donkeys. The result agrees with those obtained by (25,26) who reported that rate of infection increases in older animals. This may be because older donkeys have a higher rate of exposure to infective stages (27)

The PCR amplification of the ND2 gene produced bands of expected length (299bp) which agrees with the results obtained in previous study (20). Sequencing of amplified products showed the homology of these isolates to the reference sequence of *E.equinus* accessed in the Genbank which confirmed that equids are the specific intermediate host for *E.equinus* (1,15–21). On the other hand, (7,22) have reported that horses can be infected with *E.granulosus* s.s. (sheep strain) as well as by *E.equinus* (horse strain). Our results agreed with that obtained by (5), who confirmed that Egyptian donkeys are the main host for *E.equinus* with up to the present time no incidence of infection with other genotypes.

As the zoo's abattoir represents the main location for the slaughtering of donkeys for dietary consumption (feeding of the zoo's predator animals), we recommend the strict post mortem inspection of slaughtered donkeys, prior to introducing them as food, as well as the hygienic disposal of organs infected with HC. This will result in the breaking of the life cycle between the intermediate and the final host, with a subsequent decreasing the frequency of this disease. Furthermore, studies on hydatidosis disease in farm animals should be continued to elucidate the role of these animals in transmission of such serious disease to human populations.

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