

USE OF PCR-RFLP FOR GENETIC CHARACTERIZATION OF *ECHINOCOCCUS GRANULOSUS* ISOLATES FROM EGYPT

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ABSTRACT:

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Background: Cystic Echinococcosis (CE) is a widespread neglected zoonotic disease caused by the larval stage of the dog tapeworm *Echinococcus granulosus sensu lato* (*E. granulosus s. l.*) that occurs in most parts of the world. Egypt is considered one of the countries where CE represents a public health concern and so far, few studies were done for molecular characterization of *E. granulosus*.

Aim of the work: The aim of the present work was to use polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) targeting mitochondrial NADH dehydrogenase subunit 1 (*nad1*) for genetic characterization of Egyptian isolates of *E. granulosus* to infer the most probable transmission patterns.

Subjects and methods: Fifty Hydatid Cyst Fluid (HCF) and/or germinal layer samples (19 human, 23 camels, and 8 pigs) were collected from hydatid cysts. DNA was extracted from protoscolices and/or germinal layers from each individual cyst and used as template to amplify *nad1* gene (1071-1078 bp). The amplification products were then digested with the restriction endonuclease *Haemophilus influenza* (*HinfI*) enzyme.

Results: Two RFLP patterns were obtained, pattern I in 95.2 % of samples (12 human, 21 camel and 7 pig samples) with three fragments of 115, 218, and 738 bp and pattern II in 4.8 % (2 human samples) with two fragments of 1035 and 36 bp. In total, 85.7 % of human and 100 % of camel and pig samples shared the same digestion pattern I, while pattern II appeared exclusively in two human cases out of the 14 typed (14.3 %).

Conclusion: These results indicate that camels and pigs are crucial in the life cycle of *E. granulosus* in Egypt, although other animals may play a role.

Keywords: *Echinococcus granulosus* genotyping, *Nad1* gene, (*HinfI*) enzyme

INTRODUCTION

Echinococcus granulosus sensu lato (*E. granulosus s. l.*) larval stage (metacestode) is the causative agent of Cystic Echinococcosis (CE), which is a zoonotic disease considered to be a re-emerging disease in several countries worldwide including Africa and Middle East⁽¹⁾. CE causes great economic

loss in livestock through condemnation of infected organs (mainly liver) and can be responsible for a life-threatening infection in humans⁽²⁾. The World Health Organization (WHO) has included CE on the list of Neglected Zoonotic Diseases, for which efforts to significantly reduce transmission by 2020 are to be prioritized⁽³⁾.

E. granulosus as a species, has extensive phenotypic and genotypic variations, which vary in morphology, life cycle patterns and host specificity. The most common intermediate hosts are farm animals, such as sheep, goats, swine, camels, horses, and cattle, as well as mule deer⁽⁴⁾. Worldwide, sheep frequently present the highest infection rate and are considered the epidemiologically most relevant intermediate hosts⁽⁵⁾. While camels are the main intermediate hosts known from Sudan, the arid parts of northern East Africa, most of the Middle East and parts of central Asia and western China⁽⁶⁾. Pigs are the intermediate hosts in South America and some parts of Africa⁽⁷⁾, a small number of infections have been recorded in cattle in Sudan and Italy⁽⁸⁾.

Multiple approaches have been proposed for *E. granulosus* species differentiation including phenotypic characterization by morphology (phenotypes or phenotypic variants), intermediate host specificity, developmental rate, and infectivity to humans (biological variants), biochemical and isoenzyme characterization, or genotypic characterization (genotypes or molecular variants)⁽⁹⁾.

Although morphological and biological studies have provided extremely useful information for species identification, these features are considered variable and may be influenced by host and environmental factors and may not reflect differences at the genetic level⁽¹⁰⁾. They can also be affected by the low number of parasites and morphological similarity. So, molecular identification of *Echinococcus* species infecting humans has been developed to avoid these problems⁽¹¹⁾.

Genetic characterization of *E. granulosus* populations is crucial for the better understanding of the transmission patterns of the parasite between definitive hosts and intermediate mammalian hosts and assists significantly in the diagnosis and control of CE⁽¹²⁾. It also has a significant

impact on the taxonomy of the parasite and epidemiology of echinococcosis, as well as for the drug design and development of vaccines⁽¹³⁾. Various techniques have focused on the molecular characterization of *E. granulosus*, targeting nuclear as well as mitochondrial genes of the parasites⁽¹⁴⁾. Mitochondrial genes have been used widely in population genetics to elucidate phylogenies, as it experiences high mutation and low recombination rates and thus best reflects population genetic structure, population differentiation, and species relationships⁽¹⁵⁾. Moreover, mitochondrial DNA is reported to be more powerful than nuclear DNA within *E. granulosus* in constructing phylogenetic relationships among closely related species, because of its rapid sequence evolution⁽¹⁶⁾. Large data sets derived from mitochondrial genomes, also have the potential to resolve problematic issues in *Echinococcus* taxonomy⁽¹⁷⁾.

A variety of different molecular methods have been used to study genetic variability of *Echinococcus spp* targeting mitochondrial genome, including random amplified polymorphic DNA-PCR (RAPD-PCR)⁽¹⁸⁾, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)^(14, 19&20) and multilocus-sequence genotyping^(21&22).

Although, sequencing approach is the gold standard for genotyping assays, it is costly and cannot be practiced on a routine basis especially in developing countries, where the disease is mostly prevalent⁽¹⁹⁾. PCR-RFLP involves the specific recognition of the genome that has been purposefully chosen and reveals nucleotide variation at enzyme specific sites in the amplified fragments⁽¹⁰⁾. It is a simple and rapid method, which has minimal requirements in terms of quantity of target DNA⁽¹⁴⁾. PCR-RFLP has been used extensively to characterize strain groupings within *E. granulosus* and to detect DNA polymorphism^(10,14,17&19). However, the

results of PCR-RFLP should be indigenously interpreted, due to occurrence of unexpected mutations (nucleotide change/insertion or deletion) in parasite genome⁽¹⁴⁾.

AIM OF THE WORK:

The aim of the present study was to use PCR-RFLP targeting mitochondrial NADH dehydrogenase subunit 1 (*nad1*) for genetic characterization of Egyptian isolates of *E. granulosus* to infer the most probable transmission patterns.

SUBJECTS AND METHODS:

The study was performed on human and animal hydatid cyst fluid (HCF) samples during the period from June 2018 to August 2019. In total, 50 samples were included in the study including 19 human, 8 pigs and 23 camels. During this period, no hydatid cysts from sheep slaughtered in Cairo Abattoir were reported.

Collection of samples:

Human isolates:

They were collected from Abdominal Ultrasonography Unit of Tropical Medicine department, Kasr El-Aini Hospital, Cairo University, and from departments of Tropical Medicine, General Surgery and Cardiothoracic Surgery, Faculty of Medicine, Ain Shams University. The 19 patients had confirmed CE by HCF examination, 18 samples were collected after PAIR (Percutaneous-Aspiration-Injection-Reaspiration) technique of liver cysts. One sample was obtained after surgical removal of a pulmonary cyst.

Animal isolates:

They were collected from Cairo abattoir and consisted of 31 samples including 23 pulmonary camel and 8 hepatic pig cysts.

Parasitological study:

The HCF was examined microscopically for the presence of protoscolices to determine the cyst fertility. Protoscolices were washed several times in saline and stored at -20 °C till further use.

Molecular study analysis

DNA extraction

Genomic DNA was extracted from protoscolices using "QIAamp® DNA Mini Kit" (Qiagen, Hilden, Germany) according to manufacturer's specifications.

*PCR of the *nad1* gene*

For molecular identification, PCR amplification was performed as described by⁽²³⁾. A 1071-1078 bp fragment including the complete *nad1* gene was amplified using a forward primer: 5' TATTAAAATATTGAGTTTGCGTC-3' and a reverse primer: 5' TCTTGAAGTTAACAGCATCACGA T 3'. PCR was performed in a final volume of 50 µl containing 1x GoTaq Master Mix PCR buffer (pH 8.5), 2 mM MgCl₂, 200 µM of each dNTP, 2.5 U DNA Hot Start polymerase enzyme, 1 µM of each primer, and 10 µl of the total DNA. Thermal reactions were performed with Initial hot start at 95°C for 2 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension for 90 seconds at 72 °C. Final extension for 5 minutes at 72 °C was done. PCR products were visualized by separation on a 2% agarose gel stained with ethidium bromide. In each run negative and positive controls were included.

*PCR-RFLP of the *nad1* gene:*

Haemophilus influenza (*HinfI*) restriction endonuclease enzyme was used to digest purified PCR product of the *nad1* gene⁽¹⁹⁾. PCR products were digested for 1-3 hours according to the manufacturer's instruction (Promega). Restriction fragments were visualized by gel electrophoresis through a

2% agarose gel stained with ethidium bromide. PCR-RFLP bands were defined by their molecular weights estimated from the size standards and by using a standard curve graphed using Microsoft Excel program. Polymorphisms were scored for presence (+) or absence (-) of the bands.

Ethical consideration:

An oral consent was obtained from the patients after explaining the aim of the study to them. The study was approved by the Research Ethics Committee, Faculty of Medicine, Ain Shams University according to the regulations of Ministry of Higher Education.

RESULTS:

HCFs were collected from 19 patients and 23 camels, while germinal layers were collected from 8 pigs.

Human samples

The 19 CE human cases included 10 (52.6%) males and 9 (47.4%) females. Participants' age ranged from 10 to 60 years, with a median age of 36 years. 79% of human cases complained from right hypochondrial pain, while 21% experienced pulmonary symptoms such as coughing, chest pain or dyspnea. Regarding the site of the hydatid cysts among CE cases, 95% (18 samples) were from the liver, while 5% (one

sample) was from the lung. Microscopic examination of human HCFs in 18 (94.7%) out of the 19 human samples revealed the presence of hooks, while one sample (5.3%) was negative by microscopic examination and proved positive after germinal layer examination.

Animal samples:

Microscopic examination of HCFs of all camel samples revealed the presence of protoscolices. HCFs of all pig samples were negative and protoscolices were revealed by examination of the germinal layer

PCR-RFLP:

Amplification of the *E. granulosus* nad1 gene was successful in 46 (92 %) samples; 16 human, 22 camel and 8 pig producing the expected 1071-1078 bp band on agarose gel (figure 1), while 4 (8 %) samples; 3 human and one camel were negative. Out of the 46 successfully amplified samples, 42 were digested with *HinfI* constituting two digestion patterns (table 1). Pattern I in 95.2 % of samples (12 human, 21 camel and 7 pig samples) with three fragments of 115, 218, and 738 bp and pattern II in 4.8 % (2 human samples) with two fragments of 1035 and 36 bp (figure 2). In total 85.7 % of human and 100 % of camel and pig samples shared the same digestion pattern I, while pattern II appeared exclusively in two human cases (14.3 %) out of the 14 typed (table 2).

Table (1): RFLP patterns of nad1 of *E. granulosus* from human, camel and pig samples

Sample	N.	Restriction with <i>HinfI</i> (bp)						Digestion patterns
		36	115	218	738	1035	1071	
Human	12	-	+	+	+	-	-	I
	2	+	-	-	-	+	-	II
	2	-	-	-	-	-	+	Undigested
Camel	21	-	+	+	+	-	-	I
	1	-	-	-	-	-	+	Undigested
Pig	7	-	+	+	+	-	-	I
	1	-	-	-	-	-	+	Undigested

Table (2): Percentages of agreements of RFLP patterns from human, camel and pig samples

Digestion patterns	Isolates	Percentage
I	Human	85.7 % (12/14)
	Camel	100 (21/21)
	Pig	100 (7/7)
II	Human	14.3% (2/14)

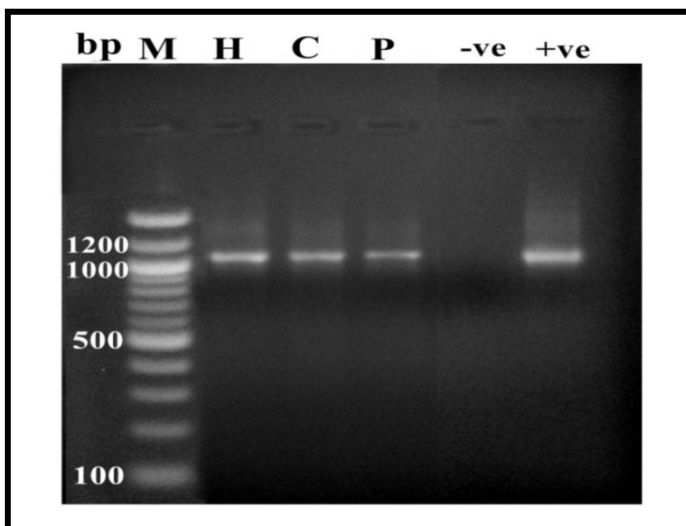


Figure (1): An Ethidium bromide stained agarose gel electrophoresis (2%) showing the PCR amplification products of *E. granulosus nad1* gene. Lane M: DNA marker (100 bp); lane H: human sample, lane C: camel sample, lane P: Pig sample, lane -ve: Negative control (without DNA template); lane +ve: Positive control of *E. granulosus* showing a DNA fragment of the expected size (1071-1078 bp).

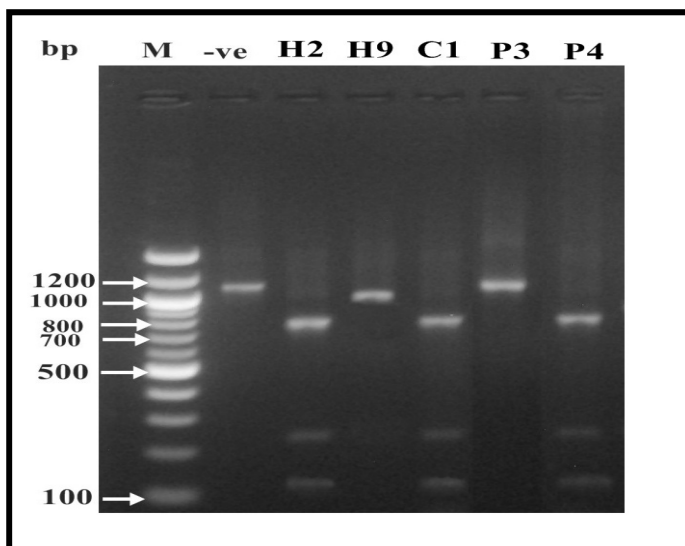


Figure (2): An ethidium bromide stained 2% agarose gel showing RFLP patterns of *E. granulosus nad1* gene after digestion with *HinI* restriction enzyme. Lane 1: (M) 100 bp-molecular marker, lane 2: -ve undigested PCR product (1071-1078 bp); lane 3: from human sample (H2) with restriction pattern I; lane 4: from a human sample (H9) with restriction pattern II (the 36 bp band is not seen in the standard gel); lane 5: from a camel sample (C1) with restriction pattern I; lane 6: from pig sample (P3) showing undigested band; lane 7: from pig sample (P4) with restriction pattern I.

DISCUSSION:

E. granulosus larval stage is the cause of CE, which is one of the most common zoonotic diseases in the populations is crucial for the better understanding of the transmission patterns of the parasite between definitive hosts and intermediate mammalian hosts and assists significantly in the diagnosis and control of CE⁽¹²⁾. In the present study, PCR-RFLP targeting the mitochondrial nad1 genetic marker was used for genetic characterization of human, camel and pig Egyptian isolates. In total 50 samples; 19 human, 8 pigs and 23 camels of confirmed CE were enrolled in the study. Amplification was successful in 46 (92 %) out of the 50 collected samples; 16 human, 22 camel and 8 pig, while 4 (8 %) samples; 3 human and one camel were negative. Targeting variable bp sequences, several investigators have amplified *E. granulosus* nad1 gene with varying success rate^(19,20,22&23). The negative results in the various studies might be due to presence of inhibitory factors in the HCF, paraffin sections and serum or technical problem in the PCR⁽²²⁾.

In the present study, two patterns were generated after digestion with *HinfI*, pattern I in 95.2 % of samples (12 human, 21 camel and 7 pig samples) with three fragments of 115, 218, and 738 bp and pattern II in 4.8 % (2 human samples) with two fragments of 1035 and 36 bp. In total, 85.7 % of human and 100 % of camel and pig samples shared the same digestion pattern I, while pattern II appeared exclusively in two human cases out of the 14 typed (14.3 %). These results indicate that camels and pigs are crucial in the life cycle of *E. granulosus* in Egypt. Although in the present study, we failed to collect hydatid cysts from other animals slaughtered in Cairo Abattoir, 14.3 % of human cases under study would probably get the infection via dogs acquired the infections from these animals.

The predominance of G6 camel strain in Egypt has been previously documented by

several investigators^(21,26,27&28). This may be due to the fact that most camels for human consumption in Egypt are imported from Sudan and are the source of *E. Canadensis* in Egypt⁽²⁷⁾. In Sudan, several studies reported the predominance of G6 in camels, goats, and cattle as well as humans in different geographical areas examined^(20&29).

In Egypt, a study using multilocus sequence typing targeting nuclear (actin II) and mitochondrial cytochrome c oxidase subunit I (cox1) and nad1 genes found G6 in 26 of the 28 camel cysts, 3 of 7 sheep cysts and the 2 buffalo derived cysts. G1 was found in one cyst from a camel and 4 of 7 cysts from sheep⁽²¹⁾. On the other hand, other researchers found G1 common in humans, camels and sheep in Egypt⁽³⁰⁾. Another study used PCR-RFLP technique based on *AluI* restriction enzyme showed that the highest genetic similarity was observed between human and sheep isolates (100%) followed by human and camel isolates, and pig and camel isolates (66.7%), while the lowest was observed between human and pig isolates (42.9 %). They reported that the sheep strain is the most relevant strain related to humans⁽³¹⁾. Genotype G1 is the most prevalent genotype worldwide, possibly due to the wide range of intermediate hosts, which facilitates higher circulation in the environment^(12, 32).

It is worth to note that 4 out of the 46 successfully amplified samples were not digested by *HinfI* restriction enzyme used, this may be due to absence of the enzyme's restriction site due to nucleotide polymorphism. The genetic diversity of G6 African and Middle Eastern isolates has been previously documented⁽³³⁾. The genetic diversity of G1 *E. granulosus* global isolates was also recorded⁽³²⁾. This result signifies the necessity of using more than one restriction enzyme to build an algorithm for the interpretation of the results of PCR-RFLP. The results should be also interpreted endogenously at the level of geographical

region or country due to occurrence of unexpected mutations (nucleotide change/insertion or deletion) in parasite genome⁽¹⁴⁾.

In conclusion, the present study as with other studies revealed that camels and pigs are crucial animals in the life cycle of *E. granulosus* in Egypt. However, other animals may play a role.

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استخدام تفاعل البلمرة التسلسلي- الرقليات للتوصيف الجيني لعزلات المشوكة الحبيبية من مصر
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المقدمة: داء الاكياس المائيه هو مرض حيواني المنشأ مهمل على نطاق واسع ناتج عن الطور اليرقي لدودة المشوكة الحبيبية (الاكينوكوكس جرانيلوساس) التي تحدث في معظم أنحاء العالم. تعتبر مصر واحدة من الدول التي يمثل فيها داء الاكياس المائيه مصدر قلق للصحة العامة ، وحتى الآن أجريت القليل من الدراسات للتوصيف الجيني للاكينوكوكس جرانيلوساس.

الهدف من البحث: دراسة التوصيف الجيني لطفيل المشوكة الحبيبية للعزلات المصرية لاستنتاج أنماط الانتقال الأكثر احتمالاً عن طريق استخدام تفاعل البلمرة التسلسلي- الرقليات (PCR-RFLP) التي تستهدف الجين الميتوكوندري nad1.

طريقة البحث: تم تجميع خمسين عينة سائل مائي من الاكياس المائيه و/ أو عينات من الطبقة الجنينية (١٩ من الانسان و ٢٣ من الأبل و ٨ من الخنازير). تم استخراج الحامض النووي من العينات المجمعة من كل كيس و استخدام تفاعل البلمرة التسلسلي- الرقليات الذي يستهدف جين nad-1 ثم بعد ذلك هضم المنتجات المتضخمة باستخدام إنزيم *HinfI*.

نتائج البحث: تم الحصول على نمطين باستخدام تفاعل البلمرة التسلسلي- الرقليات، النمط الأول ظهر في ٩٥.٢٪ من العينات (١٢ عينة بشرية، ٢١ جمل و ٧ خنزير) بثلاثة قطع ١١٥ ، ٢١٨ ، و ٧٣٨ bp والنمط الثاني في ٤.٨٪ (عينتان بشريتان) مكون من قطعتين ١٠٣٥ و ٣٦. في الجمل، شارك ٨٥.٧٪ من البشر و ١٠٠٪ من عينات الجمل والخنازير في نمط الهضم الأول، في حين ظهر النمط الثاني بشكل حصري في حالتين بشريتين من أصل ١٤ حالة تم اختبارهم (١٤.٣٪).

الاستنتاج: تشير هذه النتائج إلى أن الإبل والخنازير ضرورية في دورة حياة هذا الطفيل في مصر، على الرغم من أن الحيوانات الأخرى قد تلعب دوراً.