International Journal of Medical Arts

Volume 4, Issue 8, August 2022

https://ijma.journals.ekb.eg/

Print ISSN: 2636-4174

Online ISSN: 2682-3780

Available online at Journal Website
https://ijma.journals.ekb.eg/
Main Subject [Basic Sciences]

Original Article

Genotyping of Echinococcus Granulosus in Infected Livestock in Sharkia Governorate, Egypt

Mai Elsayed Dahroug

Department of Parasitology, Faculty of Medicine, Zagazig University, Sharkia, Egypt

Article information

Received: 05-08-2022

Accepted: 14-09-2022

DOI: 10.21608/IJMA.2022.154624.1491

Corresponding author

Email: drmai_par@yahoo.com

Citation: Dahroug ME. Genotyping of Echinococcus Granulosus in Infected Livestock in Sharkia Governorate, Egypt. IJMA 2022 August; 4 [8]: 2558-2563. doi: 10.21608/IJMA.2022.154624.1491

ABSTRACT

Background: Echinococcus granulosus enclosed a composite of various genotypes that perform distinction in the mold of the life cycle and their host categories. Thus, 10 genotypes of this parasite had been described by applying molecular approaches.

Methods: The present thesis correlates the genotypic distinction of E. granulosus metacestodes from livestock in Sharkia governorate during 2019. The study was been applied to 51 livestock organs infected with hydatid cysts. Thirty-nine samples were from sheep and 12 from buffalo. DNA was been extracted from Protoscolices [PSCs] and germinal layers of the cysts. Multiplex Polymerase Chain Reaction [m PCR] was utilized, targeting subunit 1 of mitochondrial cytochrome c oxidase [cox1] and NADH dehydrogenase 1 [nad1] genes. PCR products were isolated from the electrophoresis gel and sequenced. The sequences were compared with those related sequences available in the GenBank, using the BLAST algorithm and BioEdit software.

Results: Among 19 sheep samples, 16 [84.2 %] were from the genotype G1 while only 3 [15.8 %] samples corresponded to the genotype G1/G3. Among 4 buffalo isolates, only one [25%] was defined as G3 genotypes. Four distinct haplotypes were determined within the examined isolates from sheep and buffalo and all isolates clustered in one group.

Conclusion: The study findings demonstrated that the dominant E. granulosus in livestock isolates in Sharkia is the G1 strain [sheep strain]. Therefore, the cycle between sheep and dogs is the major cause of hydatidosis. The study's findings revealed that cooperation and control measures should be considered to prevent the disease of Sharkia. Extensive studies are been imperative to determine the dominant E. granulosus genotypes in human cases in this region.

Keywords: Echinococcus granulosus; Livestock; Genotyping; PCR.

INTRODUCTION

Cystic echinococcosis [CE] is a zoonotic parasitic disease provoked by the larval phase of Echinococcus granulosus [1]. The parasite's adult form dwells in the Canidae's intestinal tract as the definite hosts, herbivores, and humans as intermediate hosts. Infection to intermediate hosts occurs when they ingest food contaminated with the helminth eggs passed in a dog's waste [2]. The disease causes substantial economic losses in livestock and gets great mortality and morbidity in humans [3].

E. granulosus consists of complex distinct strains representing diversity in their host types and life cycle pattern [4]. Currently, there are 10 phenotypes of the parasite have been identified using the sequence of mitochondria DNA [mt DNA] by molecular methods [5]. There are four classifications of E. granulosus: ortleppi [G5], sensu stricto [G1-G3 genotypes], Canadensis [G6-G10], and equinus [G4]. All E. granulosus strains was been revealed as the cause of human CE [6]. In the Middle East, the most common genotypes in humans and livestock are G1 and G3 [7].

Middle East countries, including Egypt, were perceived as human CE endemic areas and reported the infection in many regions with a prevalence range of more than 14 % in livestock [7]. Sheep and dogs form the main disease transmission pattern in Egypt, while goats, camels, buffalo, and cattle contribute to different degrees to the parasite's life cycle [8]. Some parts of Egypt have reported instances of E. granulosus genotypes in several intermediate hosts inclusive of humans [9].

The knowledge of molecular genetics, morphological taxonomy, and evolutionary ecology of E. granulosus genotypes is required to incorporate and integrate a better understanding of the parasites' biodiversity [10]. It would be integral to determine the dominant parasite genotypes globally to provide reasonable and appropriate infection control tools [10].

METHODS

Study area: The research was been carried out in Sharkia, east of Egypt, where it has an almost warm climatic condition for livestock products and breeding, especially in farm areas. It has broad and extensive pastures with significant livestock numbers compared to other regions. The large slaughterhouses have a high daily intake capacity integral in providing livestock products and meat.

Sample Preparation: The study contributed to 51 livestock lung and liver organs infected with hydatid cysts. The samples were taken from slaughterhouses in 2019. They provided 39 samples from local sheep and 12 from local buffalo. Protoscolices [PSCs] had been taken from the fluid containing a hydatid cyst, and its sediment was frozen after being washed 3 times by using phosphate-buffered saline [PBS]. Additionally, the germinal cyst layers were carefully detached from the outer host capsules of sterile cysts. The germinal layers and PSCs were fixed in 70% ethanol to be stored at -20ºC up to microscopic and molecular examination.

Microscopic examination: The transparent hydatid fluids were aspirated and micro-scopically examined for the presence of prot-oscoleces. Hooks lengths were measured using [Olympus BX100 Microscope] and image analyzer software [Image Media Cybernetics, Germany]. Ten protoscoleces were squeezed onto a microscope slide in polyvinyl lactophenol and averages of hooks length were measured.

Genomic DNA Extraction from Isolates: As previously introduced, a DNA extraction kit [TaqMan™™ GTXpress™ Master Mix] was been used to extract the genomic DNA [gDNA] from either PSCs or germinal layers based on the manufacturers' modifications and instructions [11].

Gel Electrophoresis and Polymerase Chain Reaction: The polymerase chain reaction was applied on all 51 samples, targeting 550 bp of nad1 [12] and 450 bp fragments of cox1 [13] the gDNA, using required primers [Table 1] shows the attributes of the utilized primers and genomic regions of the targets.

The cycling parameters for amplification of the genomic pieces were: 1x [5′, 94 °C]+ 40x [45″, 95 °C+35″ 51 °C+ 45″ 75 °C]+ 1x [10′, 72 °C], 1.5% agarose gel was used to separate the PCR products, and an ultraviolet detector [Bio-Rad, USA] was used to record and visualize the ethidium bromide-stained bands.

Table [1]: Used primers for amplification of nad 1 and cox1 fragments

Genome	Primer code	Sequences
Nad 1	MS1 [F]	5'-CGT AGGTAT GTT GGT ATG TTT GGT 3′
	MS2 [R]	5'-CCA TAA TCA TAT CGC GTA CGA T- 3′
Cox 1	JB3 [F]	5'-TTATTT GGG CAT CCT GAG GTT TAT-3
	JB4.5 R]	5'-TAA AGA CAG AAC ATA ATG AAA ATG-3'

Sequencing of DNA: Based on the instructions by the manufacturer, 23 of the total 51 PCR products, nineteen sheep samples and 4 from buffalo were selected based on the resultant band quality in electrophoresis gel and Easypure Quick Gel Extrication Kit [TRANS, TransGen Biotech, South Korea] which used to purify the samples. The resulting pure isolates were sequenced for nad1 and cox1 pieces from two directions by the PCR primers. The E. granulosus sequences isolated were compared and aligned using the BLAST algorism and BioEdit program.

RESULTS

Microscopic findings: Microscopic examination revealed that most of the hydatid cysts were measured 1–8 cm in diameter, with nonsignificant variation between sizes in the sheep and cysts. By compound microscope examination, each single large or small hook consists of three parts: the blade, guard, and handle regions. Comparatively, the rostellar large and small hooks of buffalo and sheep isolates showed clear variability in their lengths, where the total length of buffalo isolates was approximately double the length of the sheep isolates [table 2].

There was a replication of target genes from the resulting PCR product when gDNA isolated from the 51 hydatid cysts were subjected to molecular assessment targeting nad1 and cox1 genomic fragments. As shown in [Figures 1&2], the 23 highest quality resulting electrophoresis gel bands of the 51 assessed samples were selected and sequenced. The resulting sequences gave accession numbers and were placed in the database of GenBank.

Sixteen [84.2 %] sheep samples were found to be of genotype G1 strain and one [5.3 %] was identified as G3 genotype strain. Besides, three [15.8%] sheep samples had no homologous to the related sequences in the GenBank. Therefore, the three sheep samples were regarded as genotypes G1/G3 strain. Nineteen samples from sheep and buffalo were homologous to the G1 E. granulosus sensu stricto [M84664], and two samples of G1 E. granulosus were [M84664]. The study's third haplotype was the only sample with similarity to G3 E. granulosus s.s [M84663]. Three sheep samples were placed in the fourth haplotype and nad 1 sequences were not present for these isolates [Table 3]. Among 4 sequenced buffalo samples, only 1 [25%] was G3 genotype while the remaining 3[75%] were G1genotype.

Table [2]: Results of microscopic and macroscopic examination of the host cysts

Host	No. of examined organs	Average hook length [µm]	Average cyst size
Sheep	39	9.5±1.72	4.5±0.7
Buffalo	12	15.8 ±0.51	4.6±0.1

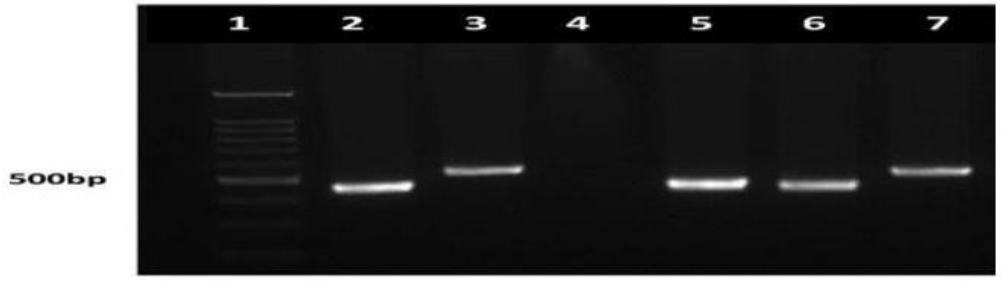

Figure [1]: Electrophoresis of PCR products using JB4 and JB3, 5 primers for cox1, MS1, and MS2 for Nad 1 on 1.5% agarose gel. LAN 1: Molecular weight marker; Lan 2: positive control for Cox1, DNA extracted from sheep isolate; Lan 3: positive control for nad 1, DNA extracted from sheep isolate; Lan 4: Negative control; Lane 5, 6: sheep isolates targeting the Cox 1 gene; Lane 7: sheep isolates targeting the Nad 1 gene

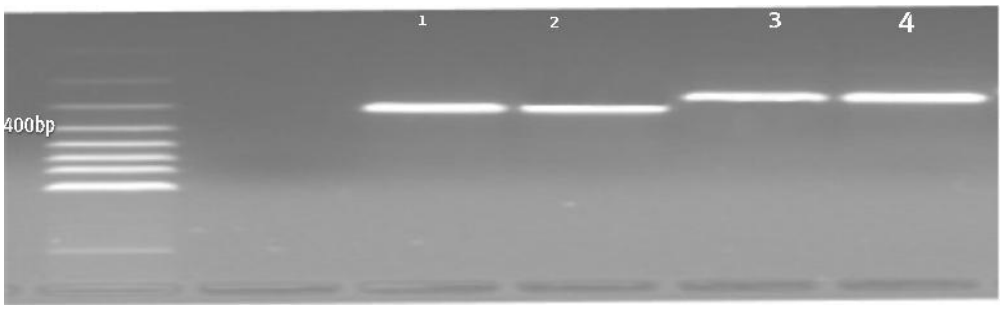

Figure [2]: Electrophoresis of PCR products. LAN 1: Negative control, Lan 2: positive control for Cox1, DNA extracted from buffalo isolate; Lan 3: positive control for nad 1, DNA extracted from buffalo isolate; Lan 4: Buffalo isolates targeting the nad1 gene

Table [3]: Echinococcus granulosus haplotypes and genotypes were detected using cox1 and nad1 sequences

Haplotypes	Number	Host			
		Sheep	buffalo	Genotypes	Homologous to
1	17	15	2	G1	M54244
2	2	1	1	G1	M84664
3	2	-	2	G3	M84663
4	2	2	-	G1/G3	-

DISCUSSION

Middle East countries had been regarded as vital foci of animal and human CE [14]. While several nuclei and mitochondrial genomes were utilized for genotyping of E. granulosus, many E. granulosus genotypes were reported in many areas in Egypt [15].

In the present study, the total hooks lengths were clear microscopic characters in separating sheep isolated from buffalo. Morphometry of the rostellar hooks is still can be considered a valid differentiating parameter in E. granulosus isolates [16]. However, its value for this purpose was been discussed in other studies [17]. This variability was been referred to as the difference in host specificity [18]. So, the validity of the analysis of rostellar hooks for strain distinction of E. granulosus can been accepted if supported by other molecular information [18].

Concerning closely related species of the E. granulosus phylogenic taxonomy, mitochondrial DNA was been identified as more efficient compared to nuclear genomes because of the large datasets sourced from the mitochondria genomes and the rapid evolution sequence. Therefore, the 12S rRNA fragment gene and the mitochondrial genes [atp6, cox1, nad5, and nad1] were studied to discriminate between different genotypes [19]. Globally, the dominant CE strains were the E. granulosus s.s [G1-G3] cases [20]. By using cox1 and nad1 genes in this study, the G1 stain was a predominant E. granulosus strain in sheep and buffalo in Sharkia. There were only two G3 strains and two G1/G3 strains established in the study.

These findings agreed with a study in Egypt by **Abd El Baki *et al***. [21] that showed that E. granulosus G1–G3 species were ascertained as the dominant genotype in intermediate hosts encompassing Buffalo, sheep, and humans. In addition, by a study on the E. granulosus genetic diversity in different hosts by **Rostami *et al***. [22], G6 and G1 genotypes were detected in goats, camels, buffalo, and sheep in Iran. **Eryıldız and Sakru** [23] in Turkey carried out a molecular study by collecting 58 human and animal E. granulosus isolates and used nad1 and cox1 DNA sequencing genes for genotyping, and nad1 and ITS1 genes for characterization. The study showed only G1 [sheep strain] and G7 [pig strain] genotypes, with the G1 strain being dominant. Finally, eight haplotypes of the Echinococcus strain were recorded during the study.

Nevertheless, nad1 and cox1 mitochondria genes have been regarded as the best and vital CE molecular characterization options and the gene cox1 gene can be utilized as an essential evolutionary marker for inter and intraspecific variant distinction [24].

In Saudi Arabia, the S12 rRNA and *cox1 gene* were used by **Al-Mutairi *et al***. to assess the novel single-nucleotide polymorphism. The researchers reported that the primary strain in

dog, sheep, and camel isolates was the G1 genotype. Thus, there is the circulation of cross-transmission of sheep-dog strain among potential definitive or intermediate hosts with *Echinococcus* heterogeneity characteristics.

In the present study, the PCR product exhibited success to replicate the target genes after the selection of nad1 and cox1 genomic fragments as targets. It has been reported that there is difficulty in the distinction between G1 & G3 genotypes cases, and those identified have been reported as either G1/G3 strain [25]. However, **Kinkar *et al*.** [26] introduced nad5 fragments for the appropriate distinction of G1 and G3 *E. granulosus* sensu stricto genotypes. **Simsek *et al*.** [27] carried out a study on E. granulosus metacedotes of sheep and camel isolates from eastern Turkey. All [100%] of the analyzed samples were established as G1-G3 strains. Another study on an animal's genetic traits and human E. granulosus isolates was been carried out in Turkey and Iran using nad1, and cox1 genes. The study revealed that the most prevalent E. granulosus was the G1 sheep strain that affected humans, sheep, and dogs in the studied regions. More so, the region was been established to contain 31 haplotype species of Echinococcus [28]. Of 112 samples, 107 had G1 strain, while five cases had G3 strain, according to a study by **Vural *et al*.** [29] Worth noting, that the G3 genotype parasites were only identified in the isolates derived from animals in the east of Turkey. The findings in this research are consistent with this study since only 2 of the samples were determined as the G3 strain and two were G1/G3, while the remaining were established as the G1 strain.

It was evident that the two evaluated livestock had similar E. granulosus genetic attributes hence camel and sheep isolates were placed in similar clusters.

Conclusions: The study findings verified that the dominant E. granulosus in livestock isolates in Sharkia is the G1 strain. The study's findings can adopt cooperation to apply the control measures and standard policies to prevent the disease in this area. Extensive studies are imperative to determine the dominant E. granulosus genotypes in human cases in Sharkia.

Conflict of interest: The author declares that no conflict of interest affects this study.

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International Journal of Medical Arts

https://ijma.journals.ekb.eg/
Print ISSN: 2636-4174
Online ISSN: 2682-3780