Veterinary Parasitology 249 (2018) 82–87

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Molecular genotyping of *Echinococcus granulosus* in the North of Iraq

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ARTICLE INFO

Keywords:
Echinococcus granulosus
Iraq
Genotyping
cox1 rrnS

ABSTRACT

Cystic echinococcosis/hydatidosis is an important cosmopolitan zoonotic disease that causes large economic losses and human suffering. The larval stages of *Echinococcus granulosus* are the etiological agents of cystic echinococcosis that showed different genotypes in different regions in the world. The present study was aimed at the detection of *E. granulosus* strains circulating in two cities from north of Iraq (Kirkuk and Sulaimania). A total of 47 specimens of hydatid cysts were collected from patients and from different domestic intermediate hosts including cattle, sheep, goat and buffalo from slaughterhouses. Molecular characterization was performed by direct sequencing of the mitochondrial DNA (mtDNA) genes coding for the cytochrome *c* oxidase I (*cox1*) and the small subunit ribosomal RNA (*rrnS*). The results showed a high prevalence for the sheep strain (G1), an isolated finding of the buffalo strain (G3) and the presence of seven and three different microvariants for *cox*1 and *rrnS*, respectively. This is the first contribution on molecular genotyping of *E. granulosus* in Iraq with the observation of genotypes other than G1.

1. Introduction

Cystic echinococcosis or hydatidosis refers to the disease due to larval stages of the cestode *Echinococcus granulosus* (Pawlowski, 1991; Lymbery and Thompson, 1996; Andresiuk et al., 2009). *E. granulosus* life cycle needs two mammals, including an intermediate host as domestic or wild ungulate, and a definitive canine host, while human is considered an accidental intermediate host (Zheng et al., 2013). Hydatidosis occurs throughout the world and causes considerable economical and public health problems in many countries (Eckert et al., 2001), since the hydatid cysts develop and grow as unilocular fluid-filled bladders into internal organs as liver and lungs of humans and other intermediate hosts (McManus et al., 2003). *E. granulosus* is widespread in all continents including circumpolar, temperate, subtropical and tropical zones. Highest prevalence of *E. granulosus* are reported from Eurasia, Africa, Australia and South America (Eckert et al., 2001). It infects at least 50 million people worldwide (Zheng et al., 2013) and it is included in the neglected tropical diseases WHO list.

Data from molecular systematics describe *E. granulosus* as a complex of species or genotypes/strains with at least five different taxonomical entities: *E. granulosus* sensu stricto (s.s.) (including genotypes G1-G3), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5), *Echinococcus canadensis* (G6-G10), and *Echinococcus felidis* ("lion strain") (Nakao et al., 2007; Hüttner et al., 2008; McManus, 2013). Endemic foci of *E. granulosus* in animals hosts associated with cystic echinococcosis cases in humans have been observed in the middle-east regions as Iraq, Iran and Gulf Littoral States (Saudi Arabia) (Eckert et al., 2001). Previous genotyping studies about *E. granulosus* strains in Iraq, performed on material collected from different intermediate hosts, have detected only the sheep strain (G1) in most of the northern regions, despite other genotypes including G4-G7 are reported (Khalf et al., 2014). Genetic analysis of molecular markers by the mean of phylogenetic or network analysis led to outline the haplotypes distribution within the G1-G2-G3 genotypes, including the genotypic variants called microvariants that can reflect differences in infectivity for distinct host groups or species, can be helpful to genetically characterize the population structure (Thompson and McManus, 2002) and to investigate the movements of haplotypes with respect to livestock domestication events (Casulli et al., 2012).

The present study was aimed to detect and characterize *E. granulosus* strains in human and domestic animals including cattle, sheep, goat and buffalo from material collected in two cities of north Iraq, Kirkuk and Sulaimania. Molecular genotyping was based on DNA sequencing of two diagnostic mtDNA genes coding for the cytochrome *c* oxidase I (*cox1*) and the small subunit ribosomal RNA (*rrnS*). Moreover, a network analysis was performed to evaluate the relationships of circulating genotypes.

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https://doi.org/10.1016/j.vetpar.2017.11.010
Received 10 August 2017; Received in revised form 30 October 2017; Accepted 20 November 2017

2. Materials and methods

2.1. Collecting of samples

A total number of 47 hydatid cysts were collected from humans and domestic slaughtered animals in Kirkuk and Sulaimania (northern Iraq). The samples included 21 specimens from cattle, 18 from sheep, 3 from goat, 2 from buffalo and 3 from humans. Hydatid cysts of human origin were collected from patients who underwent surgery in hospitals, from June of 2015 to February of 2016. The animal samples were collected from slaughterhouses in June and July 2015. Germinal layers were isolated from each cyst and washed with phosphate buffer saline (PBS), then stored in tubes with absolute ethanol until used for acid nucleic extraction.

2.2. DNA extraction

Genomic DNA was extracted using two commercial kits (Isolate II Genomic DNA Kit, Bioline, United Kingdom and Wizard Genomic DNA Purification Kit, Promega, USA) according to the manufacturer's instructions. In brief, germinative layer of individual cysts were homogenized and proteinase K digestion followed by column isolation were applied, including an internal negative control. Isolated DNA was frozen until the amplification with polymerase chain reaction (PCR) technique.

2.3. PCR amplification and gel electrophoresis

The two mtDNA target sequences *cox1* and *rrnS* genes were amplified by PCR using the following protocol: PCR was carried out using 1U of Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems, USA), 5 µl of 10X PCR buffer (Applied Biosystems), $MgCl_2$ 1 mM (Applied Biosystems), dNTPs 10 µM (Applied Biosystems), 0.5 µl of each primer (25 pmol/µl) and 5 µl of template DNA in a 50 µl final volume of reaction. Two conserved primers, JB3 (forward): 5′-TTTTTTGGGCATCC TGAGGTTTAT-3′ and JB4.5 (reverse): 5′-TAAAGAAAGAACATAATGA AAATG-3′ (Bowles et al., 1992), were used to amplify the mtDNA region corresponding to partial *cox1*. The reactions of amplification were carried out under the following conditions: a hot start 94 °C for 10 min followed by 30 cycles of 30 s denaturation at 94 °C, 40 s annealing at 52 °C, 45 s extension at 72 °C followed by a final extension of 7 min at 72 °C (Busi et al., 2007). Two conserved primers, P60 (forward): 5′-TTAAGATATATGTGGTACAGGATT AGATACCC-3′ and P375 (reverse): 5′-AACCGAGGGTGACGGGCGGTGTGTACC-3′ (Dinkel et al., 1998) were used to amplify the mtDNA region corresponding to the *rrnS* gene. The amplification was performed under the following conditions: a hot start 94 °C for 10 min followed by 30 cycles of 30 s denaturation at 94 °C, 60 s annealing at 55 °C, 60 s extension at 72 °C followed by a final extension of 7 min at 72 °C (Busi et al., 2007).

Amplicons were checked on a 1.5% agarose gel stained with GelRed solution and detected by the use of ultraviolet transillumination. Gel images were captured electronically and analyzed using Bio-Rad's Image Lab software.

2.4. Sequences analysis

SureClean (Bioline) was used according to the manufacturer's instructions for purification of positive amplicons used for sequencing analyses. Amplicons were subjected to automated sequencing by the external service Eurofins Genomics (Germany). Nucleotide sequences obtained were carefully analyzed using Chromas lite and then aligned using MEGA 7 (Kumar et al., 2016). Sequences obtained from *cox*1 and *rrn*S were then compared to GenBank retrievable sequences as reference strains and all available sequences from *cox*1 and *rrn*S genomic regions, in order to infer haplotype identity and to identify microvariants.

Homologue sequences were retrieved from GenBank and haplotypes representative of genetic variability circulating in the *E. granulosus* complex (Supplementary S1) were included with all specimens here analysed to obtain a parsimony network using TCS software (Clement et al., 2000).

3. Results

Molecular genotyping of *E. granulosus* specimens from north of Iraq was performed on a total of 47 specimens of hydatid cysts collected from different intermediate hosts and results obtained showed a positive *cox*1 amplification for 44/47 specimens with a PCR product of about 450 bp and a positive *rrn*S amplification for 39/47 specimens analysed with a PCR product of around 350 bp.

3.1. Results for cox1 sequences analysis

Dataset of partial *cox*1 region included sequences of 332 bp, obtained after manual trimming due to the different size in external ends of sequences here analyzed in comparison to the reference strains (Alignment available as Supplementary S2). Comparison was aimed to infer haplotype composition using DNAsp (Rozas et al., 2017) based on the dataset obtained in the present study, revealing the existence of 7 haplotypes. These were distributed as follow: around half sample showed G1 genotype (23/44–54.5%), one specimen showed G3 (1/44–2.3%) and the remaining specimens were distributed in 5 microvariants haplotypes of the G1/G2/G3 complex (20/44–45.5%), considering microvariants as haplotypes with few polymorphic sites in comparison to already described genotypes. Notably, an high relative frequency of the Hap5 microvariant was observed, reported in 36.4% of specimens analysed (16/44). Among the microvariants recovered, two were different from all retrievable haplotypes (Hap_7 and Hap_8). Sequences of the new haplotypes reported were submitted to GenBank under the following accession numbers XXXXX-XXXXX. With respect to city of origin, G1 and Hap5 are common in both sites of sampling (Kirkuk and Sulaimania), G3 was observed only in Sulaimania and the two new haplotypes were found in Kirkuk. Results are summarized in Table 1 and the whole alignment of partial *cox*1 sequences is available in Fig. S3. Polymorphic position of all haplotypes identified in the present investigation are available in Fig. 1.

Comparison of *cox*1 sequences here analysed with GenBank retrieved sequences led to a total of 51 haplotypes, that have been used for network inference (list of Genbank accession number is available as Supplementary S1). Correspondences were found with previous identified haplotypes circulating in several countries of middle-east regions as Turkey, Iran, Kurdistan as well as UK, Brazil and Portugal (Table 2).

Table 1
Number and relative frequencies of haplotypes for the *cox1* gene of results obtained in the current study according to the, n: number of samples. G4 and G7 strains were excluded from the table.

Host	Strain/Haplotype						
	G1 strain n (%)	G3 strain n (%)	Hap_5 n (%)	Hap_6 n (%)	Hap_7 n (%)	Hap_8 n (%)	Hap_9 n (%)
Sheep	8 (18%)	1 (2.3%)	6 (13.6)	0	1 (2.3%)	0	1 (2.3%)
Cattle	9 (20%)	0	9 (20.5%)	1 (2.3)	0	0	0
Buffalo	1 (2.3%)	0	0	0	0	1 (2.3%)	0
Goat	3 (6.8%)	0	0	0	0	0	0
Human	2 (4,5%)	0	1 (2,3%)	0	0	0	0
Total	23 (50.0)	1 (2.3)	16 (34.1)	1 (2.3)	1 (2.3)	1 (2.3)	1 (2.3)