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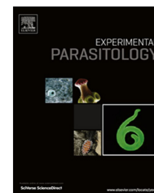

Molecular characterization of *Echinococcus granulosus* isolated from sheep in Palestine

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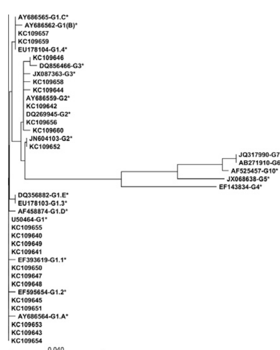
HIGHLIGHTS

- We amplified and sequenced *cox1* gene from 21 of sheep hydatid cysts.
- We found that sheep were infected with 3 genotypes G1, G2 and G3.
- The predominant genotype was G1.
- We found 2 haplotypes EG01the predominant and G1.4.
- These results may provide a preliminary data for local control programs.

GRAPHICAL ABSTRACT



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ABSTRACT

A total of twenty-three *Echinococcus granulosus* hydatid cysts were collected from infected sheep slaughtered in Nablus abattoir, Nablus – Palestine. Protoscoleces or germinal membranes were used for DNA extraction followed by PCR amplification. Amplified products were analyzed the presence of a fragment of 444 bp of the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene followed by nucleotide sequencing. Overall, 21 hydatid cysts were positive compared to a negative control. The partial sequences of *cox1* gene of *E. granulosus* strains indicated that the sheep in Palestine were infected with genotype 1 (G1), genotype 2 (G2) and genotype 3 (G3). The prevalence of these genotypes was (14/21) 66.7%, (4/21) 19.0% and (3/21) 14.3% for G1, G2 and G3, respectively. Our results showed that twelve strains of G1 belonged to the common haplotype EG01 which is the major haplotype in all the geographic populations. Phylogenetic analysis also showed that two sequences of G1 genotype which have GenBank accession No. KC109657 and KC109659 were corresponding to G1.4 micro-variants. Only the sequence of GenBank accession No. KC109652 identified in our study as G2 was found to have complete identity to the original sequence described for the *cox1* gene (GenBank accession No. M84662). It is concluded that G1 genotype is the predominant genotype in sheep in Palestine. Therefore, these findings should be taken into consideration in developing prevention strategies and control programs for hydatidosis in Palestine.

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1. Introduction

Hydatidosis is one of the most important helminthic infections causing significant morbidity and mortality in humans as well as significant economic losses in livestock animals in the Mediterranean region (Lahmar et al., 2004; Casulli et al., 2008). Palestine is one of these countries where this disease is of public health importance (Abu-Hasan et al., 2002).

The identification of *Echinococcus granulosus* variants has been carried out in different laboratories using different methods. This includes techniques based on morphology, physiology, biochemistry, immunology or molecular genetics. The techniques were more

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effective when used together (Andresiuk et al., 2009; Mowlavi et al., 2012). For example, using both morphological and molecular methods together could provide more accurate and reliable information about the extent of variation within *E. granulosus* complex. Molecular approaches especially polymerase chain reaction (PCR)-based technique, and DNA sequence analysis have been used extensively to characterize strain grouping within *E. granulosus* complex.

E. granulosus complex is a group of parasites that shows a great intra-species variability all over the world. Different molecular techniques have been used in studying this variability. Currently, genetic studies based on the partial sequences of mitochondrial cytochrome c oxidase subunit 1 (*cox1*) and NADH dehydrogenase 1 (*nad1*) genes revealed ten distinct groups called genotypes 1–10 (G1–G10) (Bowles et al., 1992; Bowles and McManus, 1993; Scott et al., 1997; Thompson and McManus, 2002; Lavikainen et al., 2003). These different genotypes include two sheep strains (G1 and G2), two bovid strains (G3 and G5), a horse strain (G4), camelid strain (G6), pig strain (G7), cervid strain (G8), human polish strain (G9) and Fennoscandian cervid strain (G10) (Grosso et al., 2012). New taxonomic revisions proposed the presence of at least 4 valid species in *E. granulosus* species complex, namely *E. granulosus* sensu stricto (*E. granulosus* s.s.), (G1–G3 genotypes), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5), *Echinococcus canadensis* (G6–G10), with leaving species status of *Echinococcus intermedius* in the G6–G10 group for further considerations (Nakao et al., 2010).

Genetic variation in *E. granulosus* may influence many different phenotypic characteristics such as the life cycle pattern, development rate, host specificity, geographical distribution, transmission dynamics, infectivity, antigenicity, control of diseases and susceptibility to chemotherapeutic agents. The study of genetic variability within and between *E. granulosus* population may have important implications for disease control program such as design and development of diagnostic reagents, vaccines and effective treatment against this parasite, pathology and epidemiology (De la Rue et al., 2006; Bhattacharya et al., 2007; Grosso et al., 2012).

In Palestine, sheep are the most common and important intermediate host for *E. granulosus*. The prevalence of infection and cyst fertility rates in sheep is high (El-Ibrahim, 2009). Due to ascertain the lack of information about hydatid DNA sequences, a preliminary pilot study was done on *E. granulosus* hydatid cyst isolates collected from sheep. This report based on sequence information seems to be the first report from Palestine

2. Materials and methods

2.1. Parasite collection

Twenty-three *E. granulosus* hydatid cysts were collected from infected sheep slaughtered in Nablus abattoir, Nablus – Palestine following standard local regulations. Cysts were processed separately under sterile conditions. In this study, an isolate represents protoscoleces or tissue of germinal layer from sterile cyst collected from an individual hydatid cyst. Protoscoleces or germinal layer tissue were rinsed in saline and then stored at −20 °C for future DNA extraction (Zhang et al., 1998).

2.2. DNA extraction

An average of 150–200 mg of cyst tissue (protoscoleces or tissue of germinal layer) was used for each DNA extraction preparation. The parasitic tissues were digested for 2 h at 57 °C with 200 µl digestion buffer (50 mM Tris–HCl, pH 8, 5 mM EDTA, 100 mM NaCl, 10% SDS) containing 20 U Proteinase K (Promega, USA). After digestion, the samples were centrifuged for 10 min at 13,000*g* and the DNA was extracted from the supernatant using phenol–chloroform method. DNA was precipitated using 96% cold ethanol (Sambrook et al., 1989). The nucleic acid pellet was washed with 70% cold ethanol, dried and then resuspended in 200 µl TE (Tris 10 mM, EDTA 1 mM, pH 8), DNA concentration was determined using spectrophotometer and the samples were stored at −20 °C until use for further DNA analysis.

2.3. PCR and DNA sequencing

Genomic DNA was amplified using specific primers for *coxI* gene, forward JB3 primer (5-TTT TTT GGG CAT CCT GAG GTT TAT-3) and reverse JB4.5 primer (5-TAA AGA AAG AAC ATA ATG AAA ATG-3 (Bowles and McManus, 1993). PCR reaction (50 µl) was performed using 2.0 U of Taq DNA polymerase, 1X PCR buffer, 2.5 mM of MgCl2, 0.2 mM of dNTPs, 0.4 µM of each primer and 150–200 ng of template DNA.

DNA amplification was performed using thermal cycler (Mastercycler Personal, Eppendorf) as the following thermal conditions: initial denaturation for 1 min at 94 °C followed by 35 cycles at 94 °C for 40 s for denaturation, annealing at 55 °C for 40 s and extension at 72 °C for 40 s. Final extension was carried out at 72 °C for 5 min. The amplified products were examined by agarose gel electrophoresis to determine the size of amplified fragment for each isolate. Amplified PCR products were purified from gel by the MinElute PCR purification kit (Qiagen, Hilden, Germany) and sequenced by dideoxy chain termination method using ABI PRISM sequencer, model 3130 (Hitachi Ltd, Tokyo, Japan), Bethlehem University, Bethlehem, Palestine. Sequence information was further submitted for accession number in primary bioinformatics web servers.

2.4. Sequence homology and phylogenetic analysis

The comparison of the continuous sequences was made with previously available sequences of the mitochondrial DNA *cox1* of *E. granulosus* genotypes in NCBI (National Center for Biotechnology Information) using BLAST system. Multiple alignments were done using ClustalW of the computer program CLC Main Workbench software (version 5.6.1, 2009, CLC bio, Aarhus, Denmark). The extent of variation amongst detected mitochondrial genotypes was estimated by pairwise comparison of nucleotide sequences with available sequence information (G1–G10) available at the NCBI database. Phylogenetic analyses were based on alignments obtained from ClustalW of a 360 bp sequence. Phylogenetic tree was constructed using the program Neighbor-Joining in the same software. The robustness of the groupings in the Neighbor Joining analysis was assessed with 1000 bootstrap resamplings.

3. Results

From a total of 23 hydatid cysts isolated from sheep, mitochondrial *cox1* gene amplification with JB3/JB4.5 primers showed that 21 (91%) isolates were positive with amplicon size of 444 bp when detected by agarose gel electrophoresis. Molecular analysis by construction phylogenetic tree showed that the sheep in Palestine were infected with at least 3 genotypes (Fig. 1), G1–G3. The prevalence of these genotypes was (14/21) 66.7%, (4/21) 19.0% and (3/21) 14.3% for G1–G3, respectively. The sequence information was between 398–444 bp, sequences were further registered at the GenBank database under the accession numbers (KC109640–KC109660).

Twelve isolates of *E. granulosus* which were identified in this study as G1, were also found to have a complete identity with

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