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# Molecular characterization of *Echinococcus granulosus* in livestock of Al-Madinah (Saudi Arabia)

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**Research Paper**

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## Abstract

*Echinococcus granulosus* is the causative agent of cystic echinococcosis, which has serious impacts on human and/or animal health, resulting in significant economic losses. *Echinococcus granulosus* comprises a number of intra-specific variants or strains at the genetic level. In Saudi Arabia, few studies were performed on genetic variations in *Echinococcus* species. Therefore, the present study aimed to investigate the phenotypic and genetic characterization of hydatid cysts harboured by sheep and camels in Al-Madinah Al-Munawarah. Samples of hydatid cysts were collected from local sheep ($n = 25$) and camels ($n = 8$). The morphological criteria of protoscoleces were investigated. To investigate the molecular characterization, random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), single-stranded conformation polymorphism (SSCP) were carried out. DNA was extracted from individual fertile cysts and subjected to RAPD-PCR analysis (using five arbitrary primers) and PCR amplification of cytochrome *c* oxidase I (*cox1*) and 12S ribosomal ribonucleic acid (12S rRNA) genes. The PCR products were subjected to SSCP analysis for genetic discrimination in *E. granulosus* isolates. In addition, partially sequencing of the mitochondrial DNA *cox1* genes was achieved for assessing the phylogenetic positions of collected isolates using some global published sequence data of *cox1* genes. The rostellar hooks of camel and local sheep isolates show remarkable variability in their dimensions. Five distinct SSCP patterns were identified in the 12S rRNA gene, showing intraspecific variations in *E. granulosus* of camels and local sheep. Sequencing of (*cox1*) genes of both local sheep and camels exhibit high similarity with those of the same gene (*E. granulosus sensu stricto*) published in NCBI BLAST.

## Introduction

Echinococcosis is a zoonotic disease of animals and humans, caused by infection with dog tapeworm *Echinococcus granulosus sensu lato* (*s.l.*) and its metacestode stage, hydatid cyst (Menekşe *et al.*, 2012). *Echinococcus granulosus* is cosmopolitan in distribution, but concentrated in the major livestock keeping or rearing areas, especially of South America, the Mediterranean, East Africa, Russia, Central Asia, China and Australia (Grosso *et al.*, 2012; Fallahizadeh *et al.*, 2019; Nungari *et al.*, 2019; Ramos-Sarmiento & Chiluisa-Utreras, 2020). In Saudi Arabia (KSA), several studies have indicated that hydatid disease is endemic where dogs are one of the major factors that distribute the disease, as well as the direct sources of human infection represented in home slaughtering of camel and sheep (Abu-Eshy, 1998; Al-Mofleh *et al.*, 2000; Adewunmi & Basilingappa, 2004; Rashed *et al.*, 2004; Al-Malki & Degheidy, 2013; Toulah *et al.*, 2017). The different intermediate hosts harbour different species of *E. granulosus*, which cause different symptoms. These extensive intraspecific variations in *E. granulosus* are associated with changes in lifecycle patterns, host specificity, geographical distribution, transmission dynamics, infectivity to human, antigenicity and sensitivity to chemotherapeutic agents (Adewunmi & Basilingappa, 2004; Eslami *et al.*, 2016).

A number of well-characterized strains of *E. granulosus* are now recognized, all of which appear to be adapted to particular lifecycle patterns and host assemblages (Thompson & McManus, 2001; McManus, 2002; Karimi & Dianatpour, 2008; Rojas *et al.*, 2017; Yan *et al.*, 2018). Currently, *E. granulosus s.l.* consists of at least five species (*E. granulosus sensu stricto* (*s.s.*), *E. equinus*, *E. ortleppi*, *E. canadensis* (G6–G10) and *E. felidis*), and one Gomo genotype of *E. granulosus* complex has been identified in Africa (Wassermann *et al.*, 2016; Romig *et al.*, 2017). Transmission cycle involving camels and dogs is responsible for human infection (Eckert *et al.*, 1989). Studying the genetic diversity of *E. granulosus* allows a clearer understanding to design more effective protocols for the management of this disease in endemic areas, especially in the field of progressive DNA vaccination using recombinant DNA



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**Table 1.** *Echinococcus granulosus* haplotypes and reference sequences used for phylogenetic analysis of partial *cox1* sequences.

| Haplotype, genotype or species | Host | Accession number (*cox1*) |
|---|---|---|
| G1 | Sheep | U50464 |
| G2 | Sheep | M84662 |
| G3 | Buffalo | M84663 |
| G4 | Horse | M84664 |
| G5 | Cattle | M84665 |
| G6 | Camel | M84666 |
| G7 | Pig | M84667 |
| G8 | Moose | AB235848 |
| G10 | Reindeer | AF525457 |
| *Echinococcus multilocularis* | Human | M84668 |
| *Echinococcus multilocularis* | Rodent | M84669 |
| *Echinococcus shiquicus* | Pika | AB208064 |
| *Echinococcus vogeli* | Rodent | M84670 |
| *Echinococcus oligarthrus* | Rodent | M84671 |
| *Echinococcus felidis* | Lion | EF558356 |
| Outgroup: *Taenia saginata* | Cattle | AB465239 |

technology, in addition to studies on vaccination resistance (Lodish *et al.*, 2000; Amini-Bavil-Olyaee *et al.*, 2006).

Few epidemiological studies have been published about *E. granulosus s.l.* in KSA, most of which focus on the seasonal prevalence and fertility degree of hydatid cysts collected from livestock (Ibrahim, 2010; Fdaladdin *et al.*, 2013; Hayajneh *et al.*, 2014; Almalki *et al.*, 2017; Amer *et al.*, 2018; Toulah & Albalawi, 2019a, b). Few studies have been conducted on the genetic diversity of *E. granulosus* in KSA (Al-Olayan *et al.*, 2014; Metwally *et al.*, 2018; Abdel-Baki *et al.*, 2018). The present study focuses on the genetic variation of hydatid cysts harboured by camels and sheep as intermediate hosts in Al-Madinah, KSA, by using random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) and single-stranded conformation polymorphism (SSCP) assays, along with sequencing the mitochondrial DNA cytochrome *c* oxidase I (*cox1*) gene for the collected specimens and assessing their phylogenetic positions using some global published sequence data of *cox1* genes (table 1).

## Material and methods

### Morphometric studies

The individual cyst was handled and processed as *E. granulosus* isolate (from camels and sheep). Cysts were dissected under sterile conditions. The clean, transparent hydatid fluids were aspirated and microscopically examined (Leica DME Binocular Microscope, Model 13595XXX, Leica Microsystems, Wetzlar (Germany)) for the presence of protoscoleces. From each isolate, ten protoscoleces were squeezed onto a microscope slide in polyvinyl lactophenol and examined for the averages of both measurements (three large and three small hooks per protoscolex) and numbers of rostellar hooks (all hooks of protoscolex were counted).

**Table 2.** The nucleotide sequences of primers used in this study and their (GC) contents.

| | Primer code | Primer sequence (5′-3′) | MWT | GC % |
|---|---|---|---|---|
| | P4 | 5′-AGACGTCCAC-3′ | 2997 | 60 |
| | P8 | 5′-GAAACACCCC-3′ | 2966 | 60 |
| | P10 | 5′-ACGCGCATGT-3′ | 3028 | 60 |
| | P12 | 5′-ACCAGGTTGG-3′ | 3068 | 60 |
| | AD4 | 5′-GTAGGCCTCA-3′ | 3028 | 60 |
| *Cox1* | JB3 | F, 5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′ | 7371 | 37.5 |
| | JB4.5 | R, 5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′ | 7459 | 20.8 |
| 12S rRNA | E.g.ss1 | F, 5′-GTA TTT TGT AAA GTT GTT CTA -3′ | 6456 | 23.8 |
| | E.g.ss1 | R, 5′-CTA AAT CAC ATC ATC TTA CCA T-3′ | 6621 | 27.3 |

MWT, Molecular weight; GC% Guanosine and Cytosine percentage.

**Table 3.** The number and hooks measurements (μm) of protoscoleces of *Echinococcus granulosus s.s.* from the camel and local sheep isolates (mean of ten protoscoleces).

| | | *Echinococcus granulosus* | |
|---|---|---|---|
| Characteristics of hooks | | Camel | Local sheep |
| Large hook | Total length | 22.3 ± 1.3 | 10.04 ± 0.22 |
| | Blade length | 10.4 ± 0.26 | 5.1 ± 0.3 |
| | Handle length | 7.7 ± 1.44 | 5.3 ± 1.42 |
| | Guard length | 3 ± 0.42 | 2.5 ± 0.16 |
| | Blade/guard distance | 10.7 ± 0.33 | 5.4 ± 0.22 |
| | **Total no. of large hooks** | **16.9 ± 0.1** | **18.80 ± 0.44** |
| Small hook | Total length | 15.4 ± 0.85 | 9.1 ± 0.17 |
| | Blade length | 8.6 ± 0.33 | 3.6 ± 0.16 |
| | Handle length | 6.4 ± 0.16 | 5 ± 0.14 |
| | Guard length | 2 ± 0.29 | 1.3 ± 0.15 |
| | Blade/guard distance | 10.1 ± 1 | 3.9 ± 0.27 |
| | **Total no. of small hooks** | **16.9 ± 0.10** | **16.4 ± 1.58** |
| **Total no. of large and small hooks** | | **34.1 ± 0.100** | **35.00 ± 0.80** |

### DNA extraction

Before DNA extraction, the hydatid fluids containing the protoscoleces were precipitated and then washed with PBS 1X. Genomic DNA was extracted by cetyltrimethylammonium bromide (CTAB) precipitation based on Yap & Thompson (1987) and Mostafa *et al.* (2003) methods, with some modifications. Briefly, tissues were homogenized individually in 1.5 ml of lysis buffer (8% Triton X-100, 0.25 M sucrose, 50 mM Tris-hydrogen chloride (HCl), 50 mM ethylenediaminetetraacetic acid (EDTA), pH

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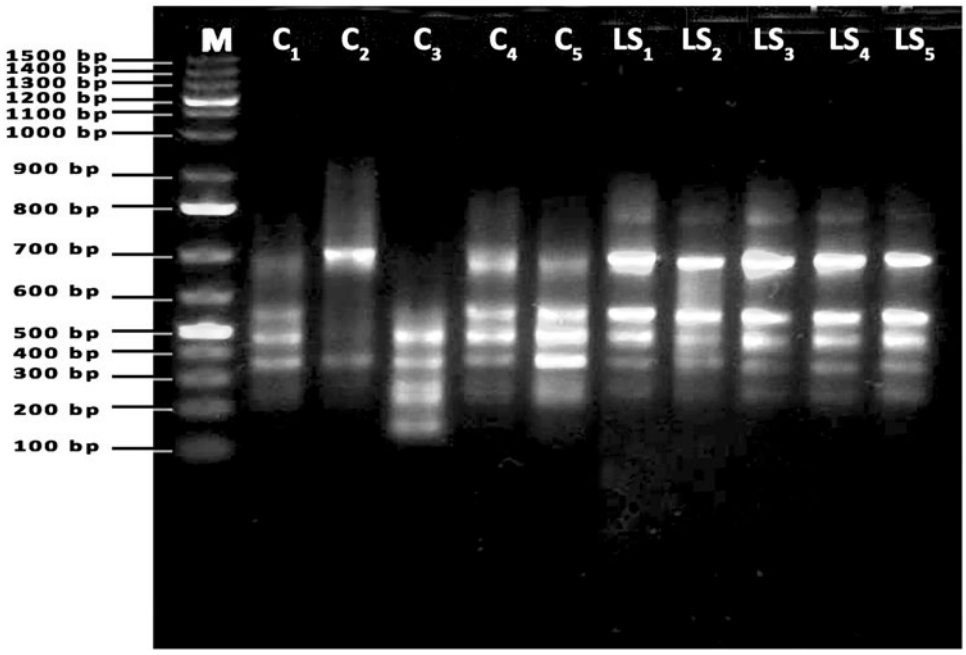


**Fig. 1.** PCR result of AD4 primer used with parasites isolated from samples of camel ($C_1$–$C_5$) and local sheep ($LS_1$–$LS_5$).

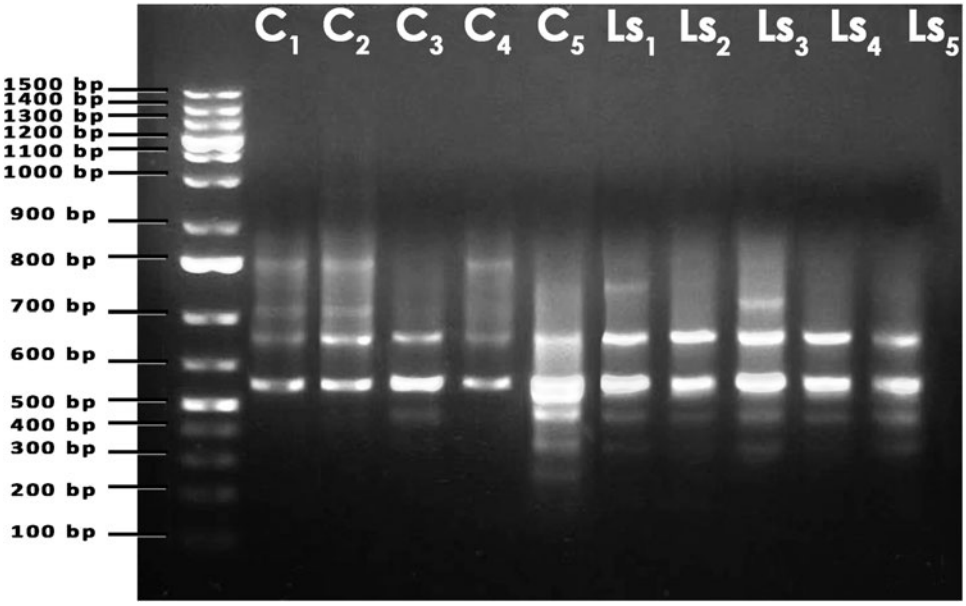


**Fig. 2.** PCR result of P4 primer used with parasites isolated from samples of camel ($C_1$–$C_5$) and local sheep ($LS_1$–$LS_5$).

7.5). Then, freshly prepared proteinase K (1 mg/ml) was added and the homogenate was incubated at 65°C in a water bath for 2 h. To precipitate the genomic DNA, 1 ml of a sterile 2% CTAB solution was added to the homogenate and centrifuged at 1500 g. The supernatant was discarded and the precipitate was dissolved in 0.5 ml of 2.5 M sodium chloride, 10 mM EDTA, pH 7.7 and diluted with 1 ml of 40 mM Tris-HCl, 2 mM EDTA, pH 7.7. Two volumes of chloroform were added to the mixture, mixed gently and centrifuged at 12,000*g* for 10 min; subsequently, the DNA was precipitated in absolute ethanol, incubated at −20 °C overnight and centrifuged at 12,000*g* for 10 min. The DNA pellet was washed with 70% ethanol and dissolved in 30 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA was stored at −20 °C until used for amplification.

### *RAPD-PCR*

PCR amplifications were performed by the protocol reported by Williams *et al.* (1990), using five arbitrary primers (table 2). The 25 µl mixture contained about 25 ng of DNA template, 1.5 unit of Taq polymerase, 10 mM deoxyribonucleotide triphosphates-deoxyribonucleotide triphosphates, 10 pM primer and 2.5 µl of

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10× PCR buffer. Amplifications were performed in a T-personal thermal cycler (Biometra, Göttingen, Germany), programmed for 45 cycles at 94°C for 1 min, 35°C for 1 min and 72 °C for 1 min. An initial denaturation step (3 min at 94 °C) and final extension holding (10 min at 72 °C) were included in the first and last cycles, respectively. Reaction products (10 µl) were resolved by 1.5% agarose gel electrophoresis at 90 V in 1× TAE buffer. The gel was stained with ethidium bromide and photographed with a digital camera (Sony, Cyper-shot, Tokyo, Japan) under an ultraviolet transilluminator. In an initial experiment, 20 decimer oligonucleotide primers were tested to determine those primers that produced reproducible RAPD patterns. Each primer was tested three times. Good and distinct patterns were produced only using five arbitrary decimers (table 2).

### PCR assay for specific primers

Two pairs of primers specific for *cox1* and 12S ribosomal ribonucleic acid (12S rRNA) genes were used for PCR assay (table 2). The PCR condition of *cox1* gene amplification was carried out according to Bowles *et al.* (1992), while that of the mitochondrial 12S rRNA gene was performed according to Stefanić *et al.* (2004). PCR products were visualized by agarose gel electrophoresis.

### SSCP technique

The SSCP method was used, as described by Zhu & Gasser (1998). First, 8 µl of PCR samples of *cox1* and 12S rRNA genes were added to 20 µl of loading dye (6X), then mixed well. After denaturation at 94 °C for 10 min and subsequent snap cooling on a frozen block (−20 °C), 8 µl of each sample was subjected to electrophoresis at 200 V for 3 h at 18 °C in a 0.4-mm-thick mutation detection enhancement gel matrix (Amersham Biosciences, New York, NY, USA). After the electrophoresis, the gel was removed and stained, then visualized over the transilluminator plate with ordinary light, and photographed using a digital camera.

### Data analysis

All observed individual bands of RAPD-PCR were scored as present or absent (1 or 0) for each isolate. The similarity coefficient (SC) was calculated according to Nei & Li (1979) and Lynch (1990) as follows: $S = 2\ Nxy/Nx + Ny$. Nxy refers to the number of bands shared between two isolates (x and y) and Nx and Ny are the number of bands amplified only by isolates x and y, respectively. Besides, the discriminatory power of each primer was calculated according to the following equation: discriminating power = the number of polymorphic bands to each primer / total number of polymorphic band of all primer X.

### Sequencing and phylogenetic analysis

*Cox1* genes of two isolates were directly sequenced from purified PCR products that were amplified using primers JB3 and JB4.5. The PCR products were purified using an ultra-clean DNA purification kit (Qiagen, Hilden, (Germany)) according to the manufacturer's protocol. Sequencing reactions were carried out with the Big Dye 3.3 terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) using the PCR conditions recommended by the manufacturer. Samples were then analysed on 6% polyacrylamide gel using an Applied Biosystems model 3100

**Table 4.** Number of total bands, polymorphic, monomorphic bands and discrimination power of RAPD primers used for *Echinococcus granulosus* diversity analysis.

| Primer | Total bands | Range of molecular size of bands | No. of polymorphic bands | | | No. monomorphic bands in camel/sheep isolates (molecular sizes) | Discriminating power of primer |
|---|---|---|---|---|---|---|---|
| | | | Camel isolate sizes (bp) | Local sheep isolate sizes (bp) | Total | | |
| AD4 | 9 | 770–150 bp | 3 (700, 280, 150) | 1 (770) | 4 | 5 (690, 550, 450, 350, 240) | 0.15 |
| P4 | 9 | 800–250 bp | 3 (800, 710, 250) | 2 (750, 720) | 5 | 4 (650, 520, 480, 350) | 0.19 |
| P8 | 10 | 690–180 bp | 5 (690, 620, 540, 340, 200) | 5 (650, 600, 500, 300, 180) | 10 | – | 0.38 |
| P10 | 10 | 790–290 bp | 3 (770, 720, 630) | 2 (790, 710) | 5 | 5 (690, 580, 530, 400, 290) | 0.19 |
| P12 | 8 | 800–290 bp | 1 (290) | 1 (480) | 2 | 6 (800, 720, 690, 540, 400, 330) | 0.07 |
| Total | 46 | | 15 | 11 | 26 | 20 | |

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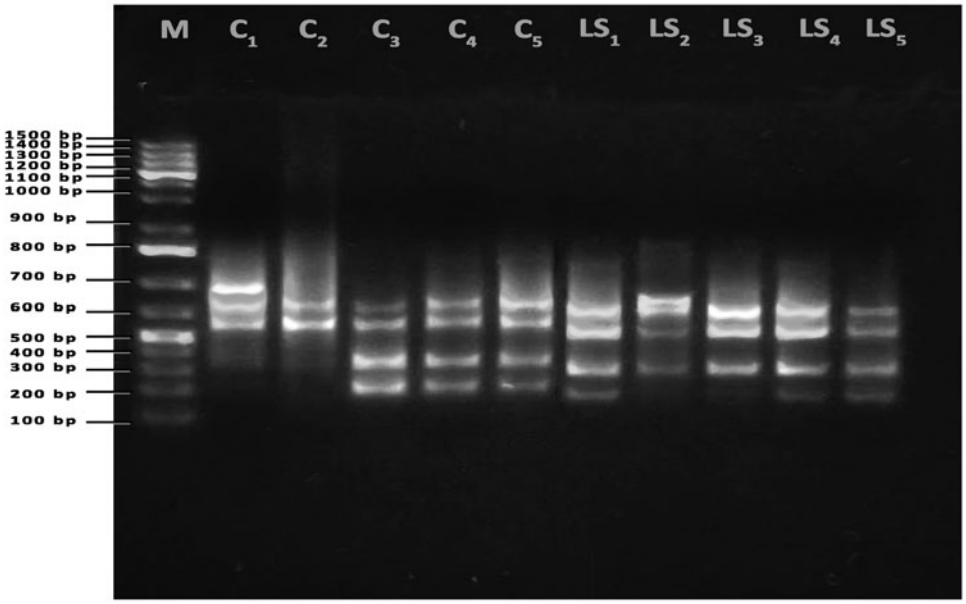


**Fig. 3.** PCR result of P8 primer used with parasites isolated from samples of camel ($C_1$–$C_5$) and local sheep ($LS_1$–$LS_5$).

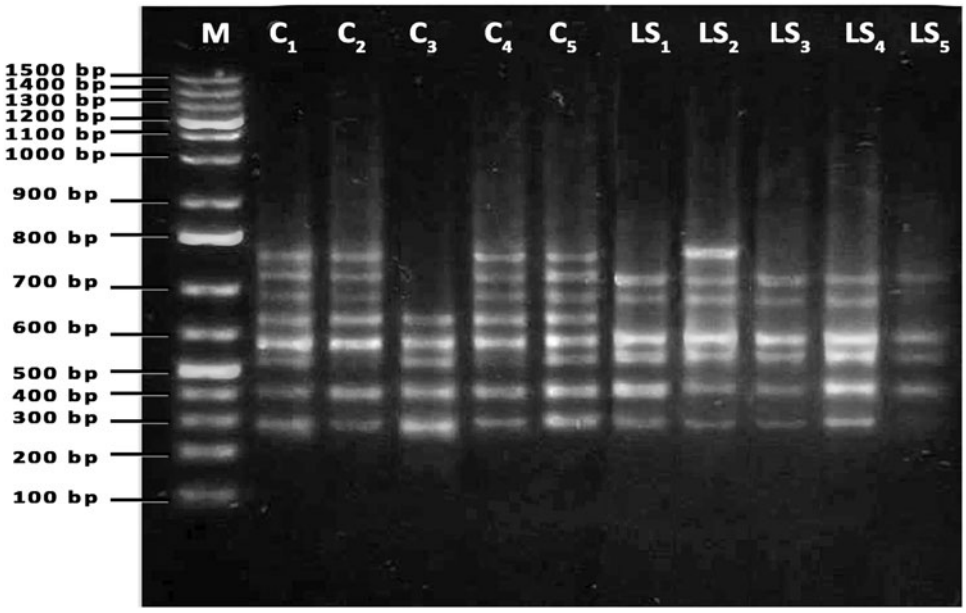


**Fig. 4.** PCR result of P10 primer used with parasites isolated from samples of camel ($C_1$–$C_5$) and local sheep ($LS_1$–$LS_5$).

Automated DNA Sequencing System. Sequences of *cox1* genes of camel and sheep isolates were separately aligned and compared with published sequences of *E. granulosus s.s.* genotypes in Basic Local Alignment Search Tool of the National Library of Medicine (NCBI BLAST) (http://blast.ncbi.nlm.nih.gov/Blast) to explore the genetic strain of *E. granulosus*. Furthermore, sequences of *cox1* genes of KSA isolates were aligned together to determine the extent of the genetic variation using BLAST® online software. The phylogenetic tree was constructed, using MEGA software version X, Pennsylvania State University, Pennsylvania, United States (https://www.megasoftware.net/), from the trimmed sequences of *cox1* genes obtained by this study, in addition to standard sequences (table 1); *Taenia saginata* was used as outgroup. The phylogenetic tree was based on the maximum-likelihood method with the *Tamura-Nei* model and bootstrap method for resampling, with the number of replicates set to 1000. The genetic distance matrix among *cox1* gene sequences of the camel and sheep isolates with some related published genotypes G1 (M84664), G2 (M84662) and G3 (M84663) was achieved (using MEGA software version X, Pennsylvania State University, Pennsylvania, United States (https://www.megasoftware.net/)) based on maximum composite likelihood.

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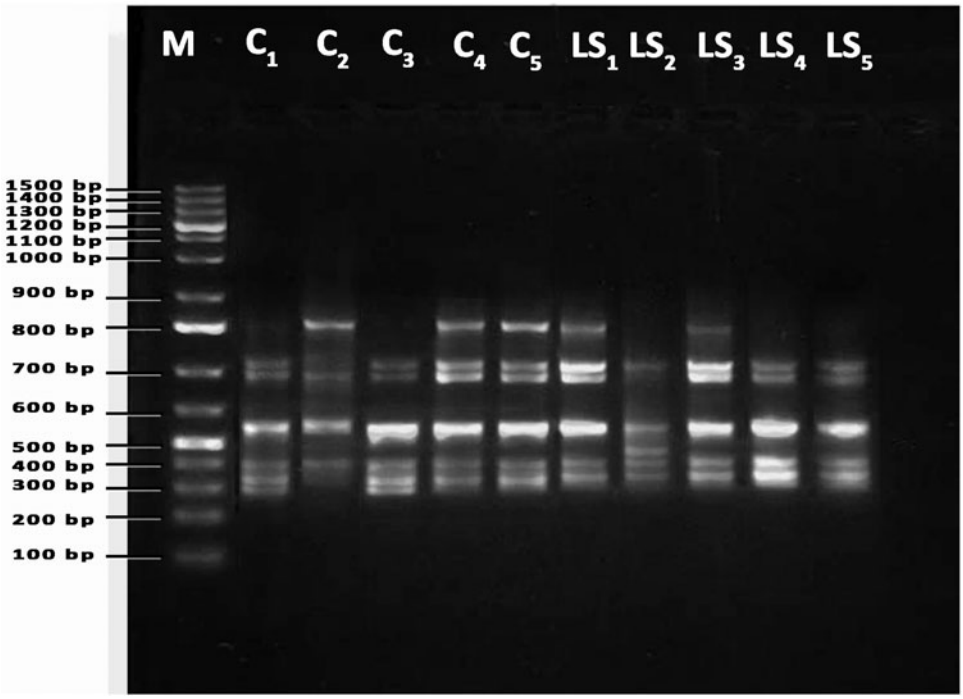


**Fig. 5.** PCR result of P12 primer used with parasites isolated from samples of camel ($C_1$–$C_5$) and local sheep ($LS_1$–$LS_5$).

**Table 5.** Average number of shared bands, similarity coefficient and genetic difference within individuals from the same/two isolates.

| Isolates | Shared bands | Similarity coefficient (mean) | Genetic difference % |
|---|---|---|---|
| Camel isolates | 18 | 52–88% (73.75%) | 26.25 |
| Local sheep isolates | 21.2 | 89–79% (84.77%) | 15.23 |
| Camel/local sheep isolates | 11.72 | 28.5–58% (47.22%) | 52.77 |

## Results

### *Morphological characters*

The morphological criteria of protoscoleces recovered from the two isolates under study were more or less similar. There were no apparent differences between protoscoleces recovered from the two isolates by stereoscope examination (low magnification). By compound microscope examination, it showed that each single large or small hook consists of three parts: the blade, guard and handle regions. Comparatively, the rostellar large and small hooks of camel and local sheep isolates show remarkable variability in their dimensions, where the total length , blade length and blade/guard distance of camel isolates were twice as many as the sheep (table 3). Non-significant variations were observed in the handle length and guard length of both the large and small hooks, as well as the total number of hooks (NH) of protoscoleces for two parasitic isolates (table 3).

### *RAPD-PCR*

All shared bands recorded between the control host tissues and hydatid cysts of each isolate were cancelled from calculations of total bands generated with five primers.

As shown in fig. 1, AD4 primer-generated bands ranged from 150 bp to 770 bp. Three DNA fragments of 700, 280 and 150 bp were only present in camel isolates, while one DNA fragment (770 bp) was present in sheep isolate. Five DNA segments of 690, 550, 450, 350 and 240 bp were common between two isolates. The primer P4 produced DNA fragments in the range from 250 to 800 bp (fig. 2). The fragments of 650, 520, 480 and 350 bp were prominent in both isolates, while the fragments of 750 and 720 bp were prominent in local sheep only. The DNA fragments of 800, 710 and 250 bp were apparent in camels, but absent in local sheep (table 4).

Primer P8 amplified bands from 180 to 690 bp. There were no generated monomorphic bands between the camel and local sheep isolates. On the other hand, each isolate produced five polymorphic bands of 690, 620, 540, 340 and 200 bp in the camel isolate and 650, 600, 500, 300 and 180 bp in the local sheep isolate (fig. 3 and table 4).

Arbitrary primer P10 amplified DNA segments ranged from 290 to 790 bp, and included five monomorphic bands of 690, 580, 530,400 and 290 bp in both camel and local sheep isolates, three bands of 770, 720 and 630 bp characteristic to camel isolate and double DNA bands of 790 bp and 710 bp characteristic to local sheep isolate (fig. 4). P12 primer-generated fragments ranged from 290 to 800 bp. Six DNA bands of 800, 720, 690, 540, 400 and 330 bp were monomorphic in two isolates; on the other hand, camel and local sheep isolates were characterized by a single band for each – 290 and 480 bp, respectively (fig. 5 and table 4).

Generally, the primer P8 is more clearly differentiating between the camel and local sheep isolates of *E. granulosus* (discriminating power 0.38), in contrast to the P12 primer, which generated approximately the same bands between the camel and local sheep isolates (table 5). In total, 20 monomorphic fragments were encountered in all primers (except P8). The lowest molecular-

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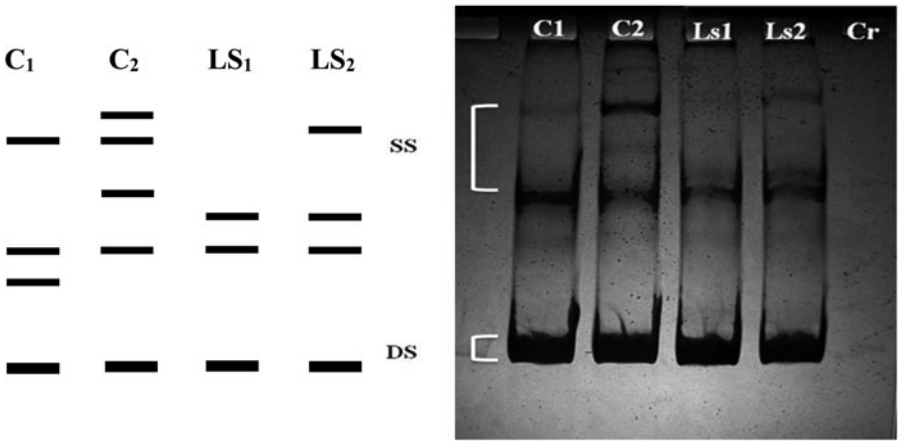


**Fig. 6.** Representative PCR-SSCP assay of mitochondrial 12S rRNA genes PCR amplified. Each PCR product was denatured and loaded in a different lane of a polyacrylamide gel. Abbreviations: SS, single-strand DNA; DS, double-strand DNA. $C_1$, $C_2$ and $LS_2$ heterozygote, while $LS_1$ homozygote.

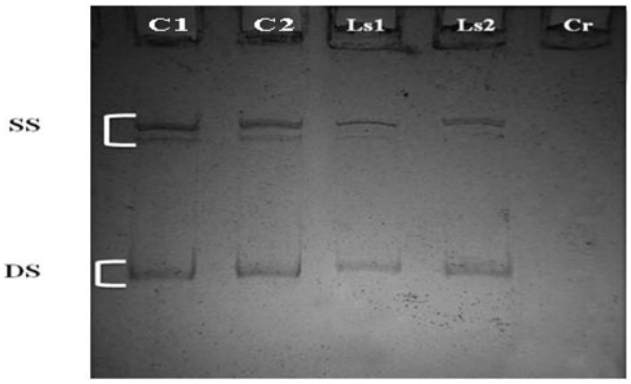


**Fig. 7.** PCR-SSCP assay of *cox1* PCR amplified. Each PCR product was denatured and loaded in a different lane of a polyacrylamide gel. Abbreviations: SS, single-strand DNA; DS, double-strand DNA.

sized band was produced in camel and local sheep isolates (150 and 180 bp) by primers AD4 and P8, respectively (table 4).

Concerning the SCs, the highest SC was among individuals of local sheep isolate (mean SC = 84.77%). On the other hand, the mean range of SCs between the camel and local sheep isolates was 47.2% (table 5).

## PCR assay and SSCP of cox1 and mitochondrial 12S rRNA genes

*Cox1* and mitochondrial 12S rRNA genes were subjected to PCR analysis on camel and local sheep isolates (two samples for each). In both host isolates, amplification of 12S rRNA genes produced a fragment of approximately 250 bp. The amplification of *cox1* genes, by contrast, showed 450–500 fragments. Although there was no variation in the size among the PCR products of mitochondrial 12S rRNA genes on agarose gel in both present isolates, SSCP analysis revealed distinct profiles for this gene between the two different *E. granulosus* isolates (fig. 6). For individual samples, 2–4 single-strand bands were resolved per lane. Multiple bands were observed because of the secondary tertiary conformation. In the camel and local sheep isolates, the most polymorphic loci were observed in the 12S rRNA gene with four alleles. This indicates an intraspecific variation in *E. granulosus* of the camels and local sheep. In the *cox1* gene, one allele was observed (fig. 7) in both the camel and local sheep isolates.

## Sequence polymorphism in cox1 gene

The *cox1* sequences showed 100% identity to published sequences of the G1–G3 complex derived from human hosts. The alignment of the *cox1* gene sequence of local sheep isolates showed the highest similarity with *E. granulosus* isolate IR.HM40 (*cox1*) gene (KF612376.1) collected from humans in Iran (direct submission), while the sequence of the *cox1* gene in the camel isolates revealed the highest similarity with the EDR1 (*cox1*) gene (HQ717148.1) collected from humans in Turkey (Eryildiz & Sakru, 2012).

Alignment between the examined *cox1* gene sequences of the local sheep and camel by Blast® software showed six sites of nucleotide variations: two deletion sites in the *cox1* gene of local sheep at positions 411 and 416, and four nucleotide substitutions ($n = 2$, A-T; A-G and T-C) at positions 380, 408, 409 and 70, respectively.

### Camel isolates

The multiple sequence alignment of camel samples with those of the referenced genotypes G1, G2 and G3 is shown in fig. 8. The camel isolates show two nucleotide substitutions (C to T and T to A at positions 70 and 380, respectively) corresponding to the G1 genotype, two nucleotide substitutions (T to C and C to T at positions 80 and 271, respectively) corresponding to the G2 genotype and three nucleotide substitutions ($n = 2$; C to T and T to C at positions 70, 271 and 80, respectively) corresponding to the G3 genotype.

### Local sheep isolates

The multiple sequence alignment of sheep samples with reference genotypes G1, G2 and G3 is shown in fig. 9. Sheep isolates show one nucleotide deletion at position 28 corresponding to genotypes G1, G2 and G3, as well as three nucleotide substitutions ($n = 2$; T to C and C to T at positions 67, 77 and 268, respectively) corresponding to the G2 genotype and two nucleotide substitutions (T to C and C to T at positions 77 and 268, respectively) corresponding to the G3 genotype. The results of the phylogenetic tree revealed that the camel and local sheep isolates were grouped in a clade with the cryptic species *E. granulosus s.s.*, with bootstrap support of 100% (fig. 10). Table 6 shows the lowest genetic distance between the sheep isolate and G3 genotype, while the camel isolate was genetically the nearest to the G2 genotype. Unique nucleotide sequences produced in this study were deposited in GenBank under accession numbers MN720281 and MN720282 for the *cox1* gene sequences of camel and sheep isolates, respectively.

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**Fig. 8.** Multiple alignment of partial *cox1* gene sequence of *Echinococcus granulosus* from camel with reference G1–G3 genotypes.

## Discussion

Rostellar hook morphology is still considered to be a valid criterion for differentiating *E. granulosus* isolates/strains (Gordo & Bandera, 1997; Harandi *et al.*, 2002; Tashani *et al.*, 2002; Ahmadi, 2004; Ahmadi *et al.*, 2006; Thompson *et al.*, 2006, Almeida *et al.*, 2007), although its usefulness for this purpose has been questioned (Sweatman & Williams, 1963; Hobbs *et al.*, 1990; Turcekova *et al.*, 2003; Ahmadi, 2004; Tang *et al.*, 2004; Hussain *et al.*, 2005; Yildiz & Gurcan, 2009). In this study, although the number of hooks in *E. granulosus* of the camel isolate was approximately similar to that of the local sheep isolate, the total length, blade and blade/guard lengths were the most variable characters. These morphometric variations could be attributed to the difference in host specificity (Lubinsky, 1962; Sweatman & Williams, 1963, Hobbs *et al.*, 1990). The validity of rostellar morphometric analysis for strain differentiation of *E. granulosus* can be accepted if supported by other molecular data (Mariaux, 1996). Many authors reported the importance of the different molecular techniques (Isozymes, protein analysis and DNA sequencing) as useful tools in differentiation among different strains of *E. granulosus* (Thompson, 1995; Harandi *et al.*, 2002).

Previously, molecular techniques were used to confirm the presence and reveal the host preferences of sheep (G1 genotype) and camel (G6 genotype) strains (Zhang *et al.*, 1998; Harandi *et al.*, 2002; Ahmadi *et al.*, 2006; Rahimi *et al.*, 2007); therefore, RAPD-PCR analysis has been used in the present study to genetically discriminate among ten samples of *E. granulosus* recovered from camels and local sheep (five from each) using five arbitrary primers. Heterogeneity was investigated between two isolates from different host species.

Genetic variations have been demonstrated among different hydatid cysts of the same host species (Lymbery & Thompson, 1989; Irshadullah & Nizami, 1997; Ahmad *et al.*, 2001; Gholami *et al.*, 2009). In the present study, five arbitrary primers indicated more intraspecific variability among individuals of *E. granulosus s.s.* in the camel isolates (less SC) if compared with that of the individuals in the local sheep isolates. This finding agrees with an Egyptian study (Taha, 2012), with a SC 92% and 87.5% in sheep and camel isolates, respectively; likewise, the SC between camel/sheep isolates in the Egyptian study was approximately equal to that of the present study: 33–45% and 47.2%, respectively.

The differences in the specific amplification of DNA fragments within camel or sheep isolates ensures the presence of heterogeneity in the examined genotype (*E. granulosus s.s.*).

In the RAPD-PCR results, primer P8 yielded band patterns that revealed a high degree of divergence between the camel and local sheep isolates. In other studies (Bhattacharya *et al.*, 2008), primers OPI-01 and OPI-15 were suitable for differentiating Indian sheep, cattle and buffalo isolates (Taha, 2012), and primers P1, P3 and OPH04 for differentiating sheep, camel, pig and donkey isolates.

Haag *et al.* (1999) demonstrated that PCR-linked SSCP provides a method to display variation between *E. granulosus s.l.* In this study, the heterogeneity within *E. granulosus* isolated from camels and local sheep was evaluated by SSCP results for PCR

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**Fig. 9.** Multiple alignment of partial *cox1* gene sequence of *Echinococcus granulosus* from local sheep with reference G1–G3 genotypes.

assay of both the 12S rRNA and *cox1* genes, which indicate intraspecific variations in the 12S rRNA gene only. Simsek *et al.* (2011) estimated the 12S rRNA gene as a tool to differentiate between the genotypes of *E. granulosus* identified based on the characteristic of SSCP profiles; furthermore, they showed that PCR-SSCP of the *cox1* gene failed in identifying the G1–G3 complex in sheep and cattle isolates and likewise, Zhang *et al.* (1990) and Oudni-M'rad *et al.* (2006). Moreover, in our findings, the 12S rRNA gene seems to show various PCR-SSCP patterns (intraspecific variation) within each isolate in both camel and local sheep. These findings support the fact that SSCP can distinguish PCR-amplified ribosomal DNA fragments, which differ by a single nucleotide (Zhu *et al.*, 1998).

Another study (Gasser *et al.*, 1998) indicates that about 75% to 100% of point mutations can be detected by PCR-SSCP over sequence lengths of 100–200 bp, but the mutation detection rate may decrease for sequences longer than 200 bp. Consequently, the heterogeneity between the present *cox1* gene sequences of the local sheep and camels (450–500 bp) cannot be detected by PCR-SSCP (although the alignment in between showed six sites of nucleotides variations). It may be possible that using more long fragments of the *cox1* gene with more genetic variation yields obvious differential patterns of PCR-SSCP.

Currently, *E. granulosus s.l.* form five valid species (*E. granulosus s.s.*, *E. felidis*, *E. equinus*, *E. ortleppi* and *E. canadensis*) (Nakao *et al.*, 2007, 2013). The present phylogenetic tree constructed using the *cox1* sequence data of KSA isolates with reference sequences showed that the local sheep and camels isolates (Al-Madinah livestock) identified as *E. granulosus s.s.* (G1–G3 complex), which comprise a deeply related complex that is distinct from other genotypes (G4–G10), as previously described by other studies (Nakao *et al.*, 2013).

This result agrees with a Saudi study (Metwally *et al.*, 2018), which reported that the sequencing of the *cox1* gene revealed the presence of cryptic species (G1–G3) in 16 of 17 sheep cysts and two of 27 camel cysts in Riyadh, KSA. Accordingly, the G1–G3 cryptic species are the most prevalent among animal isolates in KSA. This finding is similar to those of studies carried out in Iran, suggesting that G1–G3 remain the most prevalent *E. granulosus* genotypes in livestock (Nejad *et al.* 2012; Pezeshki *et al.*, 2013; Nikmanesh *et al.*, 2014).

## Conclusion

The present study enforces the relentless attempts to establish the exact haplotypes/genotypes of *E. granulosus s.l.* present in KSA livestock. Consequently, additional isolates from other hosts,

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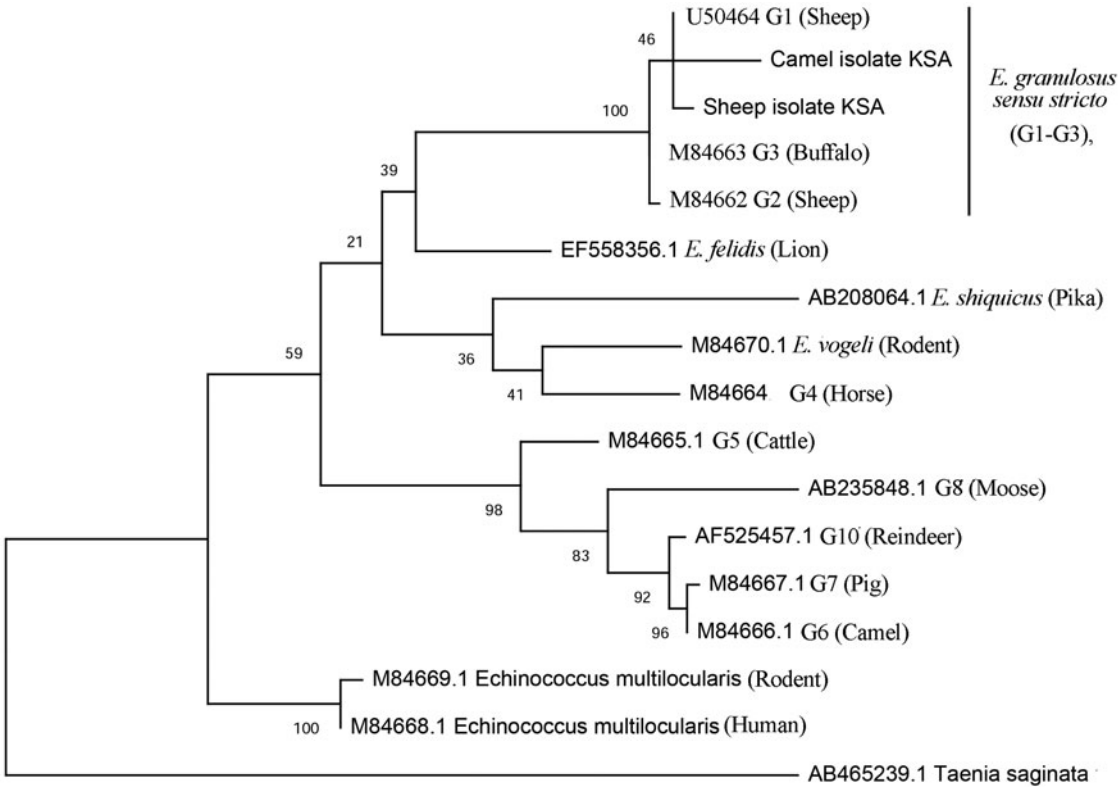


**Fig. 10.** Phylogenetic tree inferred from *cox1* genes of camel, local sheep isolates and reference sequences obtained from GenBank (based on the maximum-likelihood method).

**Table 6.** Genetic distance matrix among the camel, sheep isolates and some published genotypes G1, G2 and G3, based on maximum composite likelihood.

| | M84664 (G1) | M84663 (G3) | M84662 (G2) | Sheep isolates | Camel isolates |
|---|---|---|---|---|---|
| M84664 (G1) | | | | | |
| M84663 (G3) | 0.10995 | | | | |
| M84662 (G2) | 0.10562 | 0.00274 | | | |
| Sheep isolates | 0.11477 | **0.00552** | 0.00831 | | |
| Camel isolates | 0.19202 | 0.05413 | **0.05074** | 0.04379 | |

such as humans, cattle, goats and stray canines, and other geographic areas may be necessary to increase our understanding of the epidemiological distribution of cystic echinococcosis in KSA especially, where thousands of sheep and camels are slaughtered during the Hajj season and festival events. Moreover, for a more precise identification of *E. granulosus s.s.*, applying sequential, different molecular protocols that depend somewhat on one or more long-sequenced genes in the nuclear genome rather than the short-sequenced parts of genes in the mitochondrial genome has been recommended, alongside additional information concerning biological characteristics (host affinities, morphology).

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**Conflicts of interest.** None.

**Ethical standards.** This study was approved by the Institutional Committee of the Post-Graduate Studies and Research at Taibah University, KSA. Cystic samples were obtained from slaughtered camels and sheep, during post-mortem inspection by veterinary officers at Al-Madinah Abattoir, KSA. Formal agreement and permission for study usage of hydatid cysts were obtained from both the university and abattoir veterinarians. No experiment was conducted on live animals.

**Author contributions.**
N.M. AL-Mutairi: data curation, investigation, methodology, validation, writing (original draft). H.A. Taha: conceptualization, formal analysis, methodology, project administration, supervision, validation, writing (review and editing). A.H. Nigm: data curation, formal analysis, investigation, software, validation, writing (original draft), writing (review and editing).

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