

## Original papers

# Genotyping of human cystic echinococcosis in northeastern Iran

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**ABSTRACT.** Cystic echinococcosis (CE) is a human public health problem in northeast of Iran and there is limited information regarding the genotypes of *Echinococcus granulosus sensu lato* (*s.l.*) in human in this region. In the present study, we determined the genotypes of *E. granulosus s.l.* infecting human in northeastern Iran from Khorasan Razavi province using PCR-RFLP of rDNA ITS1 region. From April 2013 to December 2016, 50 hepatic hydatid cysts were recovered from 50 patients who underwent surgical procedure. Protoscoleces were collected from individual cysts, DNA was extracted, and the rDNA ITS1 gene was amplified by PCR and genotypes confirmed by RFLP. All the cyst materials were identified as *Echinococcus granulosus sensu stricto* (*s.s.*), indicating this species as the main cause of CE in humans in northeast of Iran.

**Key words:** human, cystic echinococcosis, genotype, PCR-RFLP

## Introduction

Cystic echinococcosis (CE), caused by the metacestode of *Echinococcus granulosus sensu lato* (*s.l.*), is a global zoonotic disease with economic importance and constitutes a threat to public health in many countries. CE is considered an emerging disease in different regions, e.g. the Middle East, central Asia, and northern and eastern Africa [1,2]. Human infection may occur after ingestion of infective eggs passed in the feces of dogs, either through direct contact or via environmental contamination. Hydatid cysts may establish in virtually all anatomic sites, but the liver and the lungs are the most frequently affected organs.

The initial phase of primary infection is always asymptomatic. After an undefined incubation period of several months or years, the infection may become symptomatic, if, cysts exert pressure on adjacent tissue and induce other pathological events. Sudden symptomatology may be due to

spontaneous or traumatic cyst rupture. The spontaneous cure is possible, due to collapse and resolution of cysts, cyst calcification or cyst rupture into the bile duct or the bronchial tree with discharge of the hydatid cyst fluids. Recurrence of the disease may occur after an operation on primary cysts.

*E. granulosus s.l.* exists as a complex of genetic genotypes that differ in their morphology, rate of development, virulence, geographic range and other factors. The new classification infers that *E. granulosus sensu stricto* (*s.s.*) groups the G1, G2 and G3 genotypes (sheep and buffalo strains), *E. equinus* (G4 genotype; horse strain), *E. ortleppi* (G5 genotype; cattle strain) [3–8]. However, there is still some uncertainty over the species status of the remaining genotypes: G6 (camel strain), G7 (pig strain), G8, and G10 (cervid strains). Thompson [4,5] suggested that the domestic strains (G6 and G7) should be regarded as a different species (*E. intermedius*) to the sylvatic strains (G8 and G10; *E. canadensis*) while Nakao et al. [6,7] proposed that



Fig.1. Geographical distribution of CE strains in different regions of Iran with showing investigated area in this study

these genotypes should be united in the species *E. canadensis*. Due to the morphological differences between the G8 and G10 genotypes, it seems appropriate to formalize the name *E. canadensis* (G10) and *E. borealis* (G8) formally in association with their morphological descriptions [9].

Among these species in *E. granulosus s.l.*, the G1 genotype is the most frequently found worldwide, produces fertile hydatid cysts mainly in sheep and is frequently isolated from humans. *E. equinus* (G4 genotype) has remarkable morphological and developmental differences with the G1 genotype and has only been found in horses and other equines and no human cases have been reported. *E. ortleppi* (G5 genotype) produces fertile cysts mainly in cattle and has been described in few human cases. Camels and goats are the main intermediate hosts for the G6

genotype, pigs for the G7 genotype and cervids for the G8 and G10 genotypes. All these genotypes have been isolated from humans [1,2].

Based on previous studies, the genetic identification of *E. granulosus s.l.* revealed *E. granulosus s.s.* and *E. canadensis* in different regions of Iran (Fig. 1) [11–13]. Moreover, different genotypes of *E. granulosus* have been reported from different livestock in this region of Iran [14–16]. The molecular identification of the occurring genotypes in human CE has noteworthy effects on control strategies in the area under the study. Therefore, the current study was conducted in northeast of Iran to determine the genotypes of different species of *E. granulosus s.l.* causing human CE.

## Materials and Methods

This study was conducted in Khorasan Razavi province that is located in northeastern Iran in the vicinity of Afghanistan and Turkmenistan with an approximate area of 173,115 km<sup>2</sup>, receives 100–500 mm of rain/year, and has air temperatures ranging between –8 and +38°C (wikipedia.org/wiki/Razavi\_Khorasan\_Province). From April 2013 to December 2016, 50 hepatic CE from 50 patients (30 male, 20 female, 20–50 years old) were collected from two central referral hospitals in Mashhad city where patients were referred for surgery. Fertility of all hydatid cysts had been confirmed microscopically by observation of protoscoleces and were transferred to the Laboratory of the Parasitology section, School of Veterinary Medicine, Ferdowsi University of Mashhad.

The protoscoleces were rinsed five times with sterile phosphate buffer (PBS, pH 7.4) and were stored in 70% ethanol until DNA isolation. Genomic DNA was extracted from protoscoleces using a commercial DNA extraction kit (MBST, Iran) according to the manufacturer's protocol. The gDNA samples obtained were stored at –20°C until further use.

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method was used for genotyping of isolates as described in our previous study [14]. The gDNA (50 ng/sample) was used for the polymerase chain reaction (PCR) amplification of the ribosomal DNA internal transcribed spacer 1 (ITS1) gene. A 462 bp fragment of the ITS1 gene was amplified by PCR using EgF (5'-CAGAGCACTTTTGTATGCA-3'), EgR (5'-ATGGTTGTTA TCG CTG CGA -3') primers.

PCR was performed in a 25 µL volume containing 2.5 mM MgCl<sub>2</sub>, 250 µM of each of the dNTPs, 20 pmol of each primer, 0.5 U Taq polymerase (Roche, Germany), 5 µL of 10× PCR buffer (Roche, Germany) and 5 µL of gDNA (50 ng/µL). Amplification conditions consists of initial denaturing step for 4 min at 95°C followed by 35 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 50°C and elongation for 45 s at 72°C and a final extension step of 10 min at 72°C. After amplification, 10 µL of the amplification products were detected on a 1.5% ethidium bromide stained agarose gel.

RFLP analysis of ribosomal DNA internal transcribed spacer 1 (ITS1) region was performed using 10 µL PCR production of each amplification

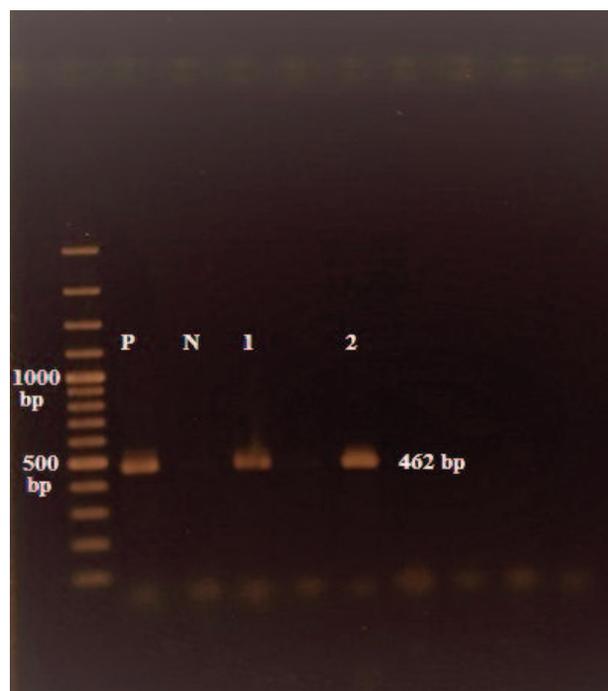


Fig. 2. Electrophoresis of PCR amplification (462 bp) provided from human sample (lane N: negative control; lane P: positive control; lane 1 and 2: positive samples). Ladder 100 bp.

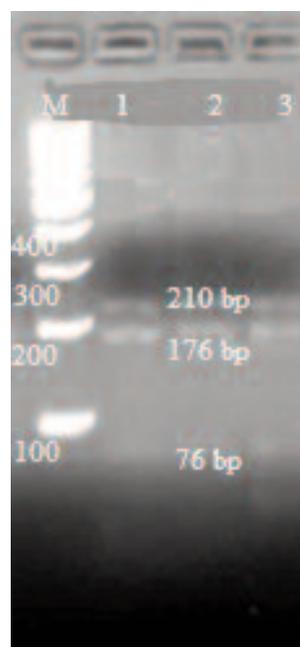


Fig. 3. Agarose gel electrophoresis of ITS1-PCR products of *E. granulosus* isolates from human after digestion with the restriction enzyme Bsh1236I. Lanes 1, 2 and 3 is G1-G3 genotype; M: ladder 100bp.

with 10 units of the restriction endonucleases Bsh1236I (5U, Fermentas) in a final volume of 20 µL for 4 hour in conditions as previously described

Table 1. Demographic characteristics of patients with hydatid cyst by sex in Khorasan Razavi Province in 2013–2016

| Variable                       | Subgroup                             | Male (30) (%) | Female (20) (%) |
|--------------------------------|--------------------------------------|---------------|-----------------|
| Location                       | urban                                | 14(46)        | 5(25)           |
|                                | rural                                | 16(54)        | 25(75)          |
| Age group                      | 10–20                                | 1(3.3)        | 0               |
|                                | 20–30                                | 8 (26.6)      | 4(20)           |
|                                | 30–40                                | 11(36.6)      | 8(40)           |
|                                | 40–50                                | 10(33.3)      | 8(40)           |
| Job                            | employed                             | 3(10)         | 1(5)            |
|                                | farmer                               | 7(23.3)       | 1(5)            |
|                                | student                              | 1(3.3)        | 2(10)           |
|                                | housekeeper                          | 0             | 11(55)          |
|                                | rancher                              | 6(20)         | 0               |
|                                | other                                | 13(43.3)      | 5(25)           |
| County                         | Mashhad                              | 4(13.3)       | 2(10)           |
|                                | Neishaboor                           | 1(3.3)        | 1(5)            |
|                                | Sabzevar                             | 1(3.3)        | 1(5)            |
|                                | Torbatjam                            | 5(16.6)       | 3(15)           |
|                                | Gonabad                              | 4(13.3)       | 2(10)           |
|                                | Fariman                              | 3(10)         | 3(15)           |
|                                | Sarakhs                              | 4(13.3)       | 3(15)           |
|                                | Kalat                                | 5(16.6)       | 4(20)           |
|                                | Ghoochan                             | 3(10)         | 1(5)            |
|                                | Contact with dog                     | Yes           | 16(53.3)        |
| No                             |                                      | 14(46.6)      | 12(60)          |
| Consumption type of vegetables | Washing with water alone             | 13(43.3)      | 10(50)          |
|                                | Washing with water and detergent     | 3(10)         | 2(10)           |
|                                | Washing with water and disinfectants | 9(30)         | 6(30)           |
|                                | All three methods                    | 5(16.6)       | 2(10)           |

[14]. Restriction fragments were electrophoresed through a 3% ethidium bromide- stained agarose gel. It should be noted that the gel be stained after electrophoresis. Five PCR products were sequenced by an ABI-3730XL capillary machine (Macrogen Inc., Seoul, Gyeonggi-do, South Korea). Sequence data were analyzed using BLAST databases from the National Center for Biotechnology.

## Results

Demographic characteristics of all studied patients by sex were shown in Table 1. All the isolates from 50 patients yielded the expected 462 bp on PCR (Fig. 2). PCR-RFLP analysis of ITS1, digested with Bsh1236I, showed that all 50 CE in human belonged to *E. granulosus s.s.* (G1–G3)

(Fig. 3). The sequences obtained from all five isolates corresponded to the G1 genotype (sheep strain). The nucleotide sequences of G1 genotype showed 100% homology with G1 sequence, which is accessible under accession number AF132700 in GenBank reference sequences.

## Discussion

In the present study, the causative agents of cystic echinococcosis in human in northeast of Iran were determined. This study provided the first comprehensive species and strain characterization of human isolates in this region, as there is no published data on the genotypes of *E. granulosus s.l.* originating from human in northeast of Iran.

Previous studies have demonstrated that the

sequences of the first and second internal transcribed spacers (ITS-1 and ITS-2) of rDNA provide reliable genetic markers among the genus *Echinococcus* [17,18]. Therefore, the ITS1 genes were used for genotyping of the *E. granulosus s.l.* via PCR-RFLP. The results of the molecular analysis using PCR-RFLP of rDNA ITS1 region revealed that G1–G3 was the dominant genotype of human CE in this region.

Human echinococcosis is one of the main public health concerns in Iran and the trend in human CE incidence in Iran from 1995 to 2014 indicated that our investigated area has the highest human CE with 1801 cases [19]. To achieve the control human CE and management programs, investigations on the epidemiology and different genotypes of parasites in the intermediate hosts should be considered in any endemic area. Different genotypes of *E. granulosus* including G1, G3 and G6 have been reported in human at different regions of Iran [11,12]. However, G1–G3 has also been considered as the most prevalent genotype in human CE [11,12,20–30]. In the current study, G1–G3 was the dominant genotype and detected in all samples. Previously, the presence of G1–G3 genotype as the dominant genotype of *E. granulosus s.l.* in sheep, goats, cattle and camel have also been reported from this area [14–16]. Furthermore, G1–G3 genotype was the only genotype isolated from dogs in Northeast of Iran [31]. The genotype G1 has the most cosmopolitan distribution and it is responsible for the great majority of human CE worldwide [32].

Moreover, the G6 genotype was reported from humans in other regions of Iran [11] and this genotype was previously recorded from goats in this region [15]. Additionally, the G7 genotype has been recorded from goats in this region of Iran [15] while pigs appear to be the principal animal intermediate host for the G7 genotype in Europe [4,32]. As the genotypes, G6 and G7 were found to be responsible for 7.34% and 3.73% of human cases of cystic echinococcosis, respectively [32]; further research will be required to determine whether these genotypes would be present in human CE in this region.

In conclusion, it seems that the *E. granulosus s.s.*, has the most important genotype in human infection with cystic echinococcosis in this district. Globally, the main intermediate host for this taxon is sheep, although infection also occurs in a wide range of livestock and herbivorous wildlife species worldwide [32]. Our data provide information

concerning the epidemiology of human CE in this region of Iran, which will likely be very significant for management and prevention plans of this disease.

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